CHEMICAL AND KINETIC CHARACTERIZATION OF GLUTAMINE HYDROLYSIS CATALYZED BY ESCHERICHIA COLI ASPARAGINE SYNTHETASE B

BY

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This work is dedicated to
my parents, Flint and Dottye Gray,
and to my husband, Richard Schnizer,
with love.

ACKNOWLEDGMENTS

I wish to thank everyone who helped me accomplish the work presented here. I would like to thank my supervisor, Dr. Sheldon Schuster, for his eagerness to discuss new ideas, his enthusiasm in my work, and for his friendship. My work benefited greatly from the guidance and helpful discussions provided by my supervisory committee: Dr. Tom O'Brien, Dr. Tom Yang, Dr. Richard Moyer, Dr. Harry Nick, and Dr. Don Allison. I would like to thank them especially for their continuing support even when my research took a change in direction. I am also indebted to Dr. Nigel Richards and Dr. Jon Stewart for their additional guidance in weekly discussions. I am especially grateful to my advisor on my first project on the hepatitis viruses, Dr. Johnson Lau, who introduced me to the fascinating world of viruses, and who had patience with me during my first few years in the laboratory.

My thanks goes to my friends in Dr. Schuster's Laboratory for providing the daily support I needed to complete this journey: Dr. Sue Boehlein, Ms. Ellen Walworth, and Mr. Gentry Wilkerson. I am especially thankful for Sue's helpful comments and ideas which helped guide my research.

Most of all I am grateful to my husband, Richard Schnizer, for all his helpful scientific advice and most of all for his love, patience, and support which helped me achieve this goal.

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ABBREVIATIONS

ALL: Acute Lymphoblastic Leukemia

AS: asparagine synthetase

AS-A: ammonia dependent asparagine synthetase A from *E.coli*

AS-B: asparagine synthetase B from *E. coli*

AMP: adenosine monophosphate

ATP: adenosine 5'-triphosphate

C1A: mutant of AS-B with cysteine-1 replaced by alanine

DON: 6-diazo-5-oxonorleucine

E. coli: Escherichia coli

GAT: glutamine amide transfer

GFAT: glutamine fructose-6-phosphate amidotransferase

GPA: glutamine 5'-phosphoribosyl-1-pyrophosphate amidotransferase

H47N: mutant of AS-B with histidine-47 replaced by asparagine

KIE: kinetic isotope effect

LGH: L-glutamic-γ-monohydroxamate

N74A: AS-B mutant with asparagine-74 replaced by alanine

PP_i: pyrophosphate

R30A: AS-B mutant with arginine-30 replaced by alanine

wt: wild-type

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHEMICAL AND KINETIC CHARACTERIZATION OF GLUTAMINE HYDROLYSIS CATALYZED BY ESCHERICHIA COLI ASPARAGINE SYNTHETASE B

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December, 1997

Chairman: Dr. Sheldon Schuster
Major Department: Biochemistry and Molecular Biology

A detailed model of glutamine hydrolysis catalyzed by *Escherichia coli* asparagine synthetase B, a class II amidotransferase, was constructed using a variety of chemical and kinetic techniques. Glutamine hydrolysis catalyzed by the class II amidotransferase family has been proposed to follow a mechanism similar to that of amide hydrolysis catalyzed by the thiol protease, papain. This study presents a comparison of the catalytic behavior of these two hydrolytic enzymes in order to determine if asparagine synthetase B-catalyzed glutaminase reaction can be described by a similar mechanism. First, this study revealed differences in acid-base catalysis between these two hydrolytic enzymes. In a continuing investigation of the importance of conserved histidines in the

glutamine utilizing domain of asparagine synthetase B, the similarity of pH profiles of wild-type and an asparagine synthetase B mutant in which a conserved histidine is replaced with an asparagine, suggests that this histidine is not likely to be involved as part of the cys-his dyad which is essential to papain-catalyzed hydrolysis. Second, this study describes the development of a model for glutamine hydrolysis catalyzed by wild-type asparagine synthetase B from a minimal mechanism. The use of such a model was confirmed by the demonstration that one main feature of papain catalysis, the formation of a thioester, was conserved in asparagine synthetase B catalyzed glutamine hydrolysis.

The values for the dissociation constants for glutamine and the inhibition constants for glutamate were determined. Furthermore, the isolation of a putative thioester intermediate provided the opportunity to measure the rate of a single step in the reaction, deacylation. These constants along with the information gained from the kinetic constants of L-glutamic-γ-monohydroxamate synthesis catalyzed by asparagine synthetase B were used in a computer simulation of the burst of glutamate production with time which showed that an additional rate limiting conformational change must be included to describe the observed kinetic behavior of wild-type asparagine synthetase B.

CHAPTER 1
INTRODUCTION

L-Asparagine and Treatment of Malignant Disease

L-asparagine was one of the first amino acids to be isolated from nature (Vauquelin & Robiquet, 1806). The importance of this non-essential amino acid in such metabolic events as the production of aspartate and ammonia (Broome, 1963a), transamination (Meister, 1952), protein synthesis (Cooney & Handschumacher, 1970), and glycosylation (Spiro, 1969) has been established. Considerable interest in L-asparagine metabolism developed after the 1953 discovery (Kidd, 1953a, 1953b) of the therapeutic effects of guinea pig serum against lymphomas in mice; and the subsequent identification of L-asparaginase, the enzyme which catalyzes asparagine hydrolysis, as the inhibitory component (Broome, 1961, 1963a, 1963b). Today, L-asparaginase is used most frequently in the treatment of acute lymphoblastic leukemia (ALL), the most common malignancy in children. When L-asparaginase is used as the sole chemotherapeutic agent, 40-68% of the patients achieve complete remission (Capizzi, et al., 1971; Uren & Handschumacher, 1977). In combination with other chemotherapeutic agents, L-asparaginase treatment results in almost 95% remission in previously untreated ALL (Sanz, et al., 1986). Despite the therapeutic potency of L-asparaginase, this treatment has several major disadvantages which

limit its use. First, L-asparaginase is toxic to organs such as the liver, pancreas, kidneys, blood clotting systems, and brain therefore, therapy is often accompanied by a wide variety of deleterious side effects (Crowther, 1971). These side effects are usually reversible and dose dependent however, in some cases they preclude the use of this drug. A second drawback to the use of L-asparaginase is that some patients who achieve remission suffer relapse with tumors resistant to the L-asparaginase therapy (Kiriyama et al., 1989; Lobel et al., 1979; Terebello et al., 1986). And finally, L-asparaginase treatment is confined to ALL because the enzyme may actually enhance the growth of some resistant tumors (Capizzi et al., 1971; Tallal et al., 1970) and, at present, there is no assay to predict sensitivity in solid tumors.

The therapeutic action of L-asparaginase is related to its ability to deplete the extracellular supply of L-asparagine (Broome, 1961; Uren & Handschumacher, 1977). While normal cells are independent of an external source of asparagine, sensitive tumor cells synthesize asparagine very slowly and are dependent on extracellular supplies of this amino acid to survive. Indeed, it has been shown that the activity of the enzyme which synthesizes asparagine, asparagine synthetase (AS), is depressed in tumor cells which are sensitive to asparaginase (Hutson et al., 1997). In contrast, increased AS activity is often observed in cell lines which exhibit a resistance to L-asparaginase (Gantt & Arfin, 1981; Kiriyama et al., 1989; Patterson & Orr, 1969) and a direct correlation between increased AS mRNA and protein content and resistance to asparaginase has been demonstrated in a human leukemia cell line (Hutson et al., 1997).

In view of the above mentioned problems associated with asparaginase therapy, alternative methods of treating ALL need to be explored. Since the effectiveness of this therapy seems to reside in reducing the circulating concentration of asparagine, inhibitors of asparagine synthetase could provide this much needed alternative. In efforts to achieve this goal, several hundred compounds have been evaluated as AS inhibitors, however none have provided both the strength and specificity required for clinical consideration (Cooney et al, 1976). Therefore, a detailed chemical and kinetic investigation of AS is essential in order to develop more effective inhibitors of this enzyme.

Asparagine Synthetase

Asparagine synthetases catalyze the ATP dependent transfer of nitrogen from either glutamine or ammonia to aspartic acid in the formation of asparagine. The biochemical and mechanistic properties of AS have been examined in a variety of sources including mammals (Hongo et al., 1978; Markin et al., 1979; Markin et al., 1981; Milman & Cooney, 1979; Van Heeke & Schuster, 1989b), fungi (Ramos & Wiame, 1979, 1980), plants (Pike & Beevers, 1982; Rognes, 1970), and bacteria (Burchall et al., 1964; Cedar & Schwartz, 1969a, 1969b; MacPhee et al., 1983) as well as in some tumor cell lines (Gantt & Arfin, 1981). In order to facilitate the recovery of sufficient amounts of AS for study, expression systems have been developed for the overexpression of human AS in yeast (Van Heeke & Schuster, 1990) and AS-B in *Escherichia coli* (Boehlein et al., 1994a).

Two classes of AS have been described which do not possess any sequence similarity. AsnA, found in many procaryotes, encodes for an AS (AS-A) which only uses ammonia as a source of nitrogen in the synthesis of asparagine (reaction 2). The kinetic and chemical mechanism of AS-A, the product of the asnA gene, has been well characterized (Cedar & Schwartz, 1969a, 1969b). In fact, the existence of an aspartyl-AMP intermediate formed during AS catalyzed asparagine synthesis was demonstrated first in experiments using AS-A (Cedar & Schwartz, 1969b). The other AS, encoded by asnB found in both prokaryotes and eukaryotes, catalyzes the synthesis of asparagine using either ammonia or glutamine as a source of nitrogen (reactions 1 and 2). These enzymes also catalyze glutamine hydrolysis (reaction 3) in the absence of the other substrates; a reaction which is stimulated by the presence of ATP.

$$\text{L-Asp} + \text{L-Gln} + \text{ATP} \rightarrow \text{L-Asn} + \text{L-Glu} + \text{AMP} + PP_i \qquad \text{(Reaction 1)}$$

$$\text{L-Asp} + NH_3 + \text{ATP} \rightarrow \text{L-Asn} + \text{AMP} + PP_i \qquad \text{(Reaction 2)}$$

$$\text{L-Gln} + H_2O \rightarrow \text{L-Glu} + NH_3 \qquad \text{(Reaction 3)}$$

Glutamine dependent asparagine synthetases are thought to be organized into two functional domains. The glutamine amide transfer (GAT) domain binds glutamine and catalyzes the hydrolysis of glutamine to glutamate and ammonia. The synthetase domain binds ATP and aspartate and catalyzes the ammonia dependent reaction as well as the transfer of nitrogen to aspartate in the glutamine dependent reaction. Evidence for the location of the two

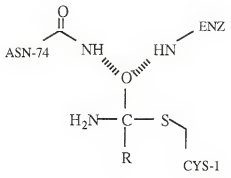
active sites in separate domains was revealed through studies using monoclonal antibodies (Pfeiffer et al., 1986, 1987) and chemical modifiers (Larsen & Schuster, 1992) that could selectively inhibit the glutaminase or asparagine synthesis activities of human or bovine AS.

While the mechanism of nitrogen transfer between the two domains is poorly understood, examination of conserved residues within the individual domains has led to the identification of binding sites and intermediates involved in the reaction. For the C-terminal synthetase domain, it has been confirmed that during asparagine synthesis, a β-aspartyl-AMP intermediate is formed which activates the aspartate carboxylate for attack by the amide nitrogen of glutamine (Luehr & Schuster, 1985). Further studies showed a binding site for aspartate located in a region highly conserved among asparagine synthetases (Boehlein et al., 1997a). And finally, another highly conserved region in the AS synthetase domain was found to contain a P-loop-like motif that is present in other enzymes which catalyze the hydrolysis of ATP to AMP and PP_i (Bork & Koonin, 1994), and has been proposed to represent a pyrophosphate binding element (Boehlein et al. manuscript in progress).

Experiments using site-directed mutagenesis have identified several conserved amino acids in the N-terminal domain that are crucial to glutamine utilization. Sequence analysis reveals that all AS contain an N-terminal cysteine residue. Milman and Cooney first proposed a critical cysteine residue in the glutamine active site when they found AS from mouse pancreas was strongly susceptible to oxidation (Milman & Cooney, 1979). More evidence for an important

cysteine residue was revealed upon the observation that bovine pancreatic AS lost glutamine dependent activity when it was covalently modified with the sulfhydryl modifier and glutamine analog, 6-diazo-5-oxo-L-norleucine (DON) (Mehlhaff & Schuster, 1991). Finally, the essentiality of the N-terminal cysteine residue was demonstrated in human AS and *E. coli* AS-B in experiments replacing cysteine-1 with either an alanine or serine residue (Boehlein et al., 1994a; Sheng et al., 1993; Van Heeke & Schuster, 1989b).

In addition to the conserved cysteine residue, the role of another completely conserved residue, asparagine-74, also has been determined. When Asn-74 was replaced with either an alanine or an aspartate, the resulting mutant enzymes (N74A & N74D) exhibited a depressed glutaminase activity (Boehlein et al., 1994b). Subsequent studies using the alternative substrate, L-glutamic acid-γ-monohydroxamate (LGH) and the AS-B mutant N74A led to the suggestion that the interaction of this asparagine residue with substrates causes a destabilization in the ES complex (Boehlein et al., 1996) and, therefore, this residue might play a role similar to that of Gln-19 of papain (Drenth et al., 1976; Menard, 1995). For papain, it is thought that Gln-19 forms an 'oxyanion hole' in the stabilization of a tetrahedral intermediate formed when the active site cysteine attacks the substrate. This 'oxyanion hole' contains a hydrogen bonding network involving the NH peptide backbone of cys-25 and the side chain NH_2 of Gln-19 which stabilizes the oxide anion bonded to the tetrahedral carbon atom. The proposed role of N74 of AS-B in forming such an oxyanion hole is illustrated below:



This proposed relationship between the thiol protease, papain, and AS-B was confirmed in studies which showed that a nitrile could act as a weak competitive inhibitor of wild type AS-B and as a substrate for the AS-B mutant N74D (Boehlein et al., 1997b); results which were consistent with similar studies using wild type and a Q19E mutant of papain (Menard, 1995).

Glutamine Amidotransferase Class II

Based on its primary amino acid sequence, asparagine synthetase is a member of a family of enzymes known as the glutamine-dependent amidotransferases. The members of this family catalyze the transfer of nitrogen from glutamine to a variety of acceptor substrates in the biosynthesis of amino acids, carbohydrates, and nucleotides (for review, Zalkin, 1993). Like AS, all amidotransferases share a modular organization with a glutamine utilizing (GAT) domain in common and a synthetase domain which is unique to each member of the family. According to the amino acid sequence alignment of the GAT domain, glutamine amidotransferases are divided into two types (Zalkin, 1993). Class I enzymes (formerly 'G-type' for the trpG gene product, anthranilate synthase) are

characterized by three catalytically important residues thought to function as a cys-his-glu catalytic triad analogous to the thiol proteases. This class of enzymes (see Zalkin, 1993 for review) includes GMP synthetase (Tiedeman et al, 1985), anthranilate synthase, (Nichols et al., 1980) carbamoyl-phosphate synthetase (Piette, 1984), imidazole glycerol-phosphate synthase (Carlomagno, 1988), aminodeoxychorismate synthase, CTP synthetase (Weng, 1986), and FGAM synthetase (Schendel et al., 1989). Asparagine synthetase as well as glutamine 5'-phosphoribosyl-1-pyrophosphate amidotransferase (GPA) (Tso et al., 1982), glutamine fructose-6-phosphate amidotransferase (GFAT) (Badet-Denisot et al., 1993), and glutamate synthase (Vanoni et al., 1991) comprise the class II amidotransferases (formerly 'F-type' for the purF gene product, glutamine 5-phosphoribosylpyrophosphate) which are characterized by a conserved N-terminal cysteine required for glutamine dependent activity.

Proposals for the Mechanism of Nitrogen Transfer

For the class II enzymes, the formation of a covalent γ-glutamyl-enzyme intermediate involving the N-terminal cysteine residue was first suggested in studies of GPA where it was shown that attachment of glutamine affinity analogs to cys-1 (Vollmer et al., 1983) or replacement with phenylalanine (Mantsala & Zalkin, 1984) caused selective elimination of the glutamine-dependent activity. Later studies using site directed mutagenesis of conserved residues in the GAT domain of GPA led to the proposal that cys-1 participated as part of a cys-his catalytic dyad in a mechanism analogous to the

thiol proteases (figure 1.1)(Mei and Zalkin, 1989). This model of nitrogen transfer involves a two step process: hydrolysis of glutamine to produce free ammonia, followed by its transfer to the acceptor substrate. In analogy to the thiol protease mechanism, a nucleophilic attack of the conserved cys-1 thiolate anion on the amide of glutamine results in a tetrahedral intermediate which, upon release of ammonia, is converted to an acylthioenzyme intermediate **1**. The released ammonia is transferred to the activated aspartate intermediate **2** in the synthetase domain for the synthesis of asparagine while the acylenzyme intermediate undergoes hydrolysis to glutamate and free enzyme via a second tetrahedral intermediate. For AS, this model for nitrogen transfer is supported by the observation that all members of its family can catalyze the synthesis of asparagine using ammonia instead of glutamine, demonstrating the presence of binding site for ammonia. Furthermore, a completely conserved cysteine has been shown to be catalytically essential in GPA (Mei & Zalkin, 1989) and AS-B (Boehlein et al., 1994a) and a residue (Asn-74) thought to play a role in forming an oxyanion hole, much like that formed by Gln-19 in papain catalysis (Menard et al. 1995), has been shown for AS-B.

While there is some evidence supporting an ammonia mediated nitrogen transfer, there are also many inconsistencies. First, was the inability to show that the class II enzymes contain a catalytically important histidine cognate to that of the thiol protease papain. Papain requires an active site histidine which functions to stabilize the cysteine thiolate anion and provide a proton to the leaving group in the conversion of the first tetrahedral intermediate to the

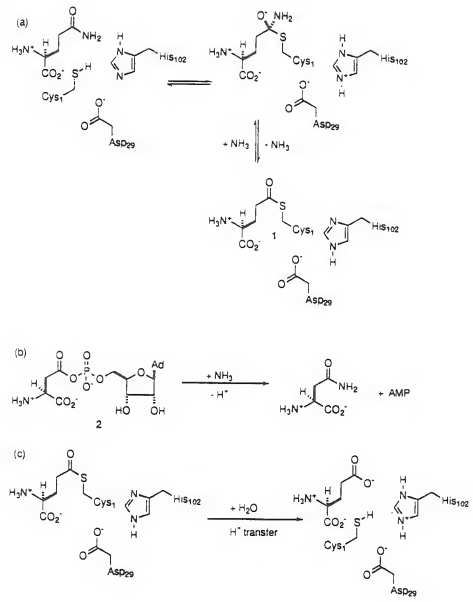


Figure 1.1 Mechanism of nitrogen transfer via an ammonia intermediate as proposed by Mei and Zalkin, 1989. (a) Mechanism for the hydrolysis of L-glutamine to yield ammonia and an acylenzyme intermediate **1** by analogy to the purF enzyme, GPA. (b) Synthesis of L-asparagine by reaction of ammonia with activated aspartyl-AMP **2**. (c) Hydrolysis of the acylenzyme intermediate to yield L-glutamate. This diagram was taken from Richards and Schuster, 1992.

thioester. Initially, it was thought, from results of site-directed mutagenesis (Mei & Zalkin, 1989), that His-102 was involved in such acid-base catalysis for GPA and covalent modification studies using diethyl pyrocarbonate suggested that GFAT (Badet-Denisot & Badet, 1992) also possessed this important histidine. However, site-directed mutagenesis of each of the conserved histidines in the GAT domain of AS-B revealed little or no change in the kinetic parameters (Boehlein et al., 1994a). Subsequent structural studies of GPA (Kim et al., 1996; Smith et al., 1994) and the N-terminal domain of GFAT (Isupov et al., 1996) did not reveal a histidine in the appropriate position to participate in acid-base catalysis. Alternatively, these structural studies led to the proposal that the α-amino group of cys-1 serves as the general acid-base catalyst (Smith, 1995). Subsequent structural comparisons of GPA with other hydrolytic enzymes placed the class II enzymes into a larger superfamily called the N-terminal nucleophile (Ntn) hydrolases which includes the 20S proteasome and penicillin acylase (Brannigan et al., 1995; Duggleby et al., 1995; Seemuller et al., 1996). This family is characterized by an N-terminal catalytic nucleophile which is thought to be activated by proton transfer from its side chain to the free N-terminus (Seemuller et al., 1996). However, experiments aimed at establishing the functional role of the N-terminal amine using mutants of GPA and GFAT with an additional N-terminal residue have yielded contradictory results (Isupov et al., 1996; Kim et al., 1996).

Further evidence against an ammonia mediated nitrogen transfer in AS-B catalyzed asparagine synthesis was revealed in an investigation of the kinetic isotope effects (KIE) (Stoker et al. 1996)

for AS-B catalyzed glutamine dependent reactions. The KIEs associated with placing ^{15}N in the primary amide of glutamine were examined for both glutamine dependent reactions and the resulting values were interpreted using ^{15}N KIE determinations for papain catalyzed peptide hydrolysis (O'Leary et al, 1974). For papain, the KIE value was shown to be consistent with a mechanism where the first irreversible step is C-N bond cleavage to form the thioester intermediate (O'Leary et al., 1974). It was expected that C-N bond cleavage would be rate limiting in the AS-B catalyzed glutaminase reaction as well and thus give the same ^{15}N (V/K) isotope effect as papain. Furthermore, an ammonia mediated nitrogen transfer would require a common pathway for glutamine hydrolysis in the two glutamine dependent reactions. Therefore, if AS-B followed an ammonia mediated mechanism, it was expected that the ^{15}N KIEs determined for both glutamine dependent reactions would be identical. Surprisingly, the glutaminase ^{15}N (V/K) was significantly smaller than that obtained for papain catalyzed peptide hydrolysis, indicating that a step other than C-N bond cleavage may be rate limiting. Moreover, the KIE for glutaminase was smaller than that for the glutamine dependent synthesis reaction. These results were regarded as the first direct evidence against the mediation of nitrogen transfer by enzyme-bound ammonia.

In addition to the above mentioned inconsistencies between AS-B catalytic behavior and an ammonia mediated nitrogen transfer, questions addressing the difficulties of maintaining the free ammonia in the active site in its unprotonated form led to the proposal of alternative mechanisms for nitrogen transfer (Figure 1.2) (Richards &

Schuster, 1992). The first proposal for an alternate mechanism of nitrogen transfer involved the formation of an imide intermediate (Richards & Schuster, 1992). According to this hypothesis, a direct nucleophilic attack of the primary amide of glutamine on the β-aspartyl-AMP intermediate **2** to form an imide intermediate **3**. Reaction of this intermediate with cys-1 causes the release of asparagine and the formation of a thioacylenzyme intermediate **1** which forms free enzyme and glutamate in a mechanism similar to the ammonia mediated mechanism. An attempt to isolate such an intermediate was made using the AS-B mutant (C1A) which lacks the cysteine required to catalyze C-N bond cleavage of the imide intermediate. However, an imide intermediate was not detected by HPLC using chemically synthesized imide as a standard, when C1A AS-B was incubated with glutamine, ATP, and aspartic acid. It was concluded that participation of an imide intermediate in nitrogen transfer catalyzed by AS-B was unlikely (Boehlein et al., unpublished observations).

In a second alternative hypothesis for the mechanism of nitrogen transfer (Stoker et al., 1996), Cys-1 reacts with glutamine to form the first tetrahedral intermediate **5** (figure 1.3). In contrast to the ammonia mediated mechanism, however, the tetrahedral intermediate makes a subsequent direct attack on the β-aspartyl-AMP intermediate to form an intermediate in which glutamine is covalently linked to β-aspartyl-AMP **6**. Cleavage of the C-N bond in this intermediate releases the acylenzyme and forms a second tetrahedral intermediate **8**. Asparagine is then formed from a loss of

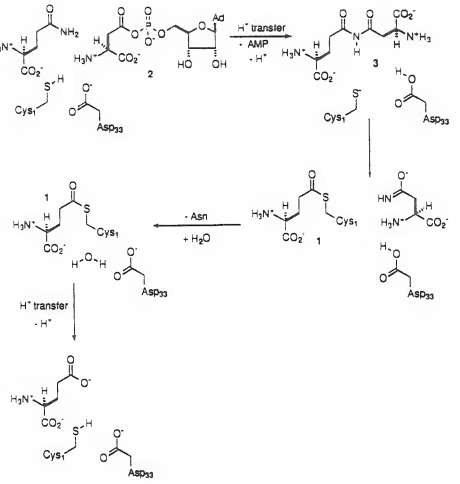


Figure 1.2 Mechanism of nitrogen transfer via an imide intermediate **3** as proposed by Richards and Schuster, 1992. Attack of the primary amide occurs directly on the activated L-aspartate **2**. Cys-1 and Asp-33 are then involved in the hydrolysis of the imide to yield L-glutamate and L-asparagine in subsequent steps.

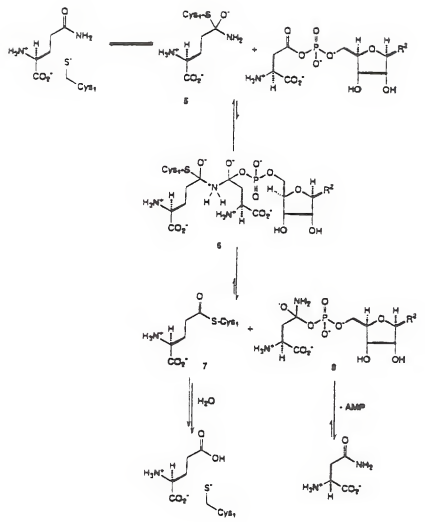


Figure 1.3 Mechanism of nitrogen transfer via direct transfer of the nitrogen from glutamine to aspartyl-AMP as proposed by Stoker et al., 1996).

AMP from the tetrahedral intermediate. Interestingly, this mechanism would not necessarily require N-protonation before C-N bond cleavage in the glutamine dependent synthesis reaction and, therefore, may explain the differences in the ^{15}N (V/K) isotope effects for the two glutamine dependent reactions.

Despite the progress that has been made in understanding the functions of the individual domains of AS, the mechanism of nitrogen transfer remains unequivocally defined. One difference between the mechanism involving free ammonia and that of a direct attack is the requirement, in the former, of a common mechanism for glutamine hydrolysis in the glutaminase and glutamine dependent asparagine synthesis reactions. Therefore, it is important to understand the mechanistic details of both the glutamine dependent synthesis and glutamine hydrolysis in order to discriminate between the two mechanisms. The ability of AS-B to catalyze the hydrolysis of glutamine in the absence of the other amino acids provides the opportunity to develop a model of the glutaminase reaction from which to identify the individual steps and intermediates in the reaction as well as examine the functional roles played by the conserved amino acids in the GAT domain. In efforts to begin building such a model, this dissertation focuses on the chemical and kinetic mechanisms of glutamine hydrolysis catalyzed by AS-B. Differences and similarities in the mechanism of substrate hydrolysis catalyzed by AS-B and papain will be examined through pH analysis of AS-B catalyzed glutaminase activity and by isolation of a covalent intermediate in the reaction pathway of glutamine hydrolysis catalyzed by AS-B. A variety of kinetic techniques will be used to

determine the individual rate constants of the glutaminase reaction and these will be used to build a model of glutamine hydrolysis catalyzed by AS-B.

CHAPTER 2
pH DEPENDENCE OF THE GLUTAMINASE REACTION CATALYZED BY ASPARAGINE SYNTHETASE B

Introduction

Asparagine Synthetase B (AS-B) catalyzes the ATP dependent synthesis of asparagine using aspartic acid and either glutamine or ammonia as a nitrogen source (reactions 1 and 2). In the absence of aspartate and ATP, this enzyme also is capable of catalyzing glutamine hydrolysis (reaction 3).

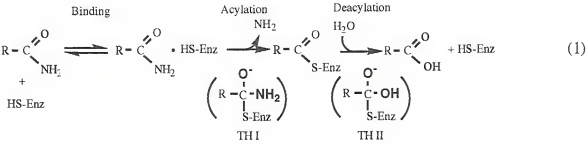
$$\text{L-Gln} + \text{ATP} + \text{L-Asp} \rightarrow \text{L-Asn} + \text{AMP} + PP_i + \text{L-Glu} \quad \text{(reaction 1)}$$

$$NH_3 + \text{ATP} + \text{L-Asp} \rightarrow \text{L-Asn} + \text{AMP} + PP_i \quad \text{(reaction 2)}$$

$$\text{L-Gln} + H_2O \rightarrow \text{Glu} + NH_3 \quad \text{(reaction 3)}$$

Amino acid sequence alignment of the N-terminal glutamine utilizing domain of AS-B indicates that this enzyme is a member of the class II glutamine amidotransferase family (Zalkin, 1993) which includes glutamine 5'-phosphoribosylpyrophosphate amidotransferase (GPA) (Tso et al., 1982), glutamine fructose-6-phosphate amidotransferase (GFAT) (Badet-Denisot & Badet, 1993), and glutamate synthase (Vanoni et al., 1991). Like AS-B, members of this family of enzymes catalyze the transfer of the amide nitrogen of glutamine to various acceptor molecules including amino acids, nucleotides, and carbohydrates.

The mechanism of nitrogen transfer remains controversial and poorly understood. Results from studies using site-directed mutagenesis suggested the catalytic importance of a conserved N-terminal cysteine, a histidine, and an aspartic acid in glutamine utilization in GPA (Mei & Zalkin, 1989). This observation led to the hypothesis that glutamine amidotransferases catalyze glutamine hydrolysis by a mechanism similar to that of amide hydrolysis catalyzed by the thiol proteases, typified by papain (for review see Brocklehurst et al., 1987), as shown below (1).



(1)

In analogy to the papain mechanism, a nucleophilic attack of the N-terminal cysteine on the side-chain amide of glutamine forms a tetrahedral intermediate (TH I). Protonation of the amide allows C-N bond cleavage to produce a thioester intermediate and free ammonia. The ammonia is then transferred to the acceptor molecule and the thioester intermediate is broken down, via another tetrahedral intermediate (TH II), to produce glutamate and free enzyme. As illustrated above, an active site cysteine and an acid-base catalyst (a role played by his-159 in papain) are vital components to such a reaction scheme. The cysteine is involved in the activation of glutamine as described above. In papain, the histidine is thought to

play three essential acid-base roles including 1) stabilization of the thiolate; 2) donation of a proton to the amide of the first tetrahedral intermediate to increase its leaving group ability; and finally 3) activation of water for a nucleophilic attack on the thioester intermediate by accepting a proton (Storer & Menard, 1994).

This mechanism of ammonia mediated nitrogen transfer is supported by the ability of the amidotransferases to use ammonia as a nitrogen source and thus the demonstration of an ammonia binding site. Furthermore, the presence of a completely conserved N-terminal cysteine, which has been shown to be essential for glutamine dependent activity of GPA (Mei & Zalkin, 1989) as well as both human and *E. coli* asparagine synthetases (Boehlein et al., 1994a; Sheng et al., 1993; Van Heeke & Schuster, 1989b), suggests that this residue may function in a manner similar to that of the active site cysteine of the thiol proteases. On the other hand, the presence of an important histidine in the active site of the class II enzymes has become increasingly doubtful. While the studies of GPA mentioned above (Mei & Zalkin, 1989) and chemical modification studies of GFAT (Badet-Denisot & Badet, 1992) seemed to indicate the presence of an essential histidine in these enzymes, site-directed mutagenesis of residues, known to be conserved among asparagine synthetases, did not reveal any histidines required for activity in AS-B (Boehlein, et al., 1994a). Moreover, recent structural studies of GPA (Kim et al., 1996; Smith et al., 1994) and the glutamine utilizing domain of GFAT (Isupov et al., 1996) suggested that there were no histidines in position to act as part of a cys-his dyad. Structural comparisons later revealed that the protein fold of GPA (Brannigan et

al., 1995) was homologous to that of a family of N-terminal nucleophile (Ntn) hydrolases which also includes penicillin acylase (Duggleby et al., 1995), the 20S proteasome (Lowe et al., 1995; Seemuller et al., 1996), and aspartylglucosamidase(Fisher et al., 1993; Oinonen et al. 1995). It is thought that members of the Ntn family use the N-terminal Ser, Thr, or Cys as the nucleophilic catalyst and the N-terminal α-amino group as the acid-base catalyst. However, whether or not the class II amidotransferases use the N-terminal amino group in acid-base catalysis remains uncertain as results from experiments which have placed an extra amino acid at the N-terminus of GPA (Kim et al., 1996) have been inconclusive.

Further investigation is necessary in order to understand fully how acid-base catalysis occurs during the glutamine hydrolysis catalyzed by the class II amidotransferases. In fact for AS-B, the possibility of an essential histidine still remains. A computer model of a hypothetical AS-B glutamine binding site, constructed using the crystallographic coordinates for the covalent adduct of DON and *E. coli* GPA, revealed that histidine-47 of AS-B was in an appropriate position to function as part of a cys-his dyad cognate to that of papain (Richards & Schuster, 1997). Moreover, this histidine is partially conserved in asparagine synthetases and a histidine in a comparable position is found in several other class II amidotransferases (Figure 2.1), thus its importance cannot be overlooked. In an effort to gain a clearer understanding as to how acid-base catalysis occurs during glutamine utilization by AS-B, it is the purpose of this chapter to explore the glutaminase activity of this

```
LtHmAS     CGIWAL< >HRGPD< >FGFHRLAV< >NGEIYNHKAL
GnHmAS     CGIWAL< >HRGPD< >FGFHRLAV< >NGEIYNHKAL
RatAS      CGIWAL< >HRGPD< >FGFHRLAV< >NGEIYNHKAL
MurAS      CGIWAL< >HRGPD< >FGFHRLAV< >NGEIYNHKAL
HumAS      CGIWAL< >HRGPD< >FGFHRLAV< >NGEIYNHKKM
SoyAS      CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
LotAS      CGILAV< >HRGPD< >LAHQRLAI< >NGEIFNHEEL
FavaAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
PeaNAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
AlflAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEDL
PeaRAS     CGILAV< >HRGPE< >LAQQRLAI< >NGEIYNHEDL
AspaAS     CGILAV< >HRGPD< >LSHQRLAI< >NGEIYNHEEL
ArabAS     CGILAV< >HRGPD< >LAHQRLAV< >NGEIYNHEEL
BrssAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
RiceAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
MaizAS     CGILAV< >HRGPD< >LAHQRLAI< >NGEIYNHEEL
ScerAS     CGIFAA< >HRGPD< >FVHERLAI< >NGEIYNHIQL
CeleAS     CGVFSI< >HRQPD< >LVHERLAI< >NGEIVNHGEL
EcoAS      CSIFQV< >HRQPD< >LAHERLSI< >NQEIYNHQAL
            1         29        47        74     80
ScerGA     CGILGI< >HRGQD< >FTQQRVS.< >NGNLVNTASL
HumGS      CGIFAY< >YRGYD< >HKQQDMDL< >NGIITNYKDL
ScerGS     CGIFGY< >YRGYD< >.TKQNPNR< >NGIITNFREL
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Figure 2.1: Sequence alignment of part of the GAT-domains of known asparagine synthetases and a partial set of class II amidotransferases showing highly conserved residues. Numbering is according to the amino acid sequence of AS-B. Sequence alignments taken from Richards & Schuster, 1997. LtHmAS = *Cricetulus longicaudatus* AS; GnHmAS = *Mesocricetus auratus* AS; RatAS = *Rattus norvegicus* AS; MurAS = *Mus musculus* AS; HumAS = *Homo sapiens* AS; Soy AS = *Glycine max* AS; Lot AS = *Lotus japonicus* AS; Fava AS = *Vicia faba* AS; PeaNAS = *Pisum sativa* AS (root); AspaAS = *Asparagus officinalis* AS; ArabAS = *Arabidopsis thaliana* AS; BrssAS = *Brassica oleracea* AS; RiceAS = *Oryza sativa* AS; MaizAS = *Zea mays*; Scer AS = *Saccharomyces cerevisiae* AS; CeleAS = *elegans* AS; EcoAS = *Escherichia coli* AS; ScerGA = *Saccharomyces cerevisiae* GPA; HumGS = *Homo sapiens* GFAT; ScerGS = *Saccharomyces cerevisiae* GFAT.

enzyme as a function of pH. In order to address the possibility that H47functions in an acid-base capacity, the effects of mutations at this position on the pH-rate profile will be characterized.

Materials and Methods

Chemicals and Reagents

Restriction and modifying enzymes were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Other chemicals, including L-glutamine and L-glutamic-γ-monohydroxamate, were obtained from SIGMA Chemical Company (St. Louis, MO) and were of the highest commercial purity. Oligonucleotide primers were synthesized on an Applied Biosystems 380B DNA synthesizer by the DNA Synthesis Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Polymerase chain reactions (PCR) were performed on an Ericomp (San Diego, CA) thermocycler using the GeneAmp DNA Amplification Reagent Kit with AmpliTaq from Perkin-Elmer. Double stranded DNA sequencing was performed using the U.S. Biochemical Corp. Sequenase 2.0 Sequencing kit.

Bacterial Strains and Plasmids

Bacterial strains BL21DE3pLysS (F^-, ompT, rb^-, mb^-), supplied by Studier and Moffat; and NM522 (supE, thi (lac-proAB), hsd5, (r^-m^-)/F' pro AB, lac I^q Z M15), obtained from Stratagene (La Jolla, CA), are both derivatives of *Escherchia coli* K-12. The plasmid pBluescript was obtained from Stratagene (La Jolla, CA) and the plasmid pETB has been described previously (Boehlein et al., 1994a). *E. coli* host cells were transformed according to the procedures of Hanahan (Hanahan, 1983).

Enzyme Preparation

Construction of recombinant wild-type AS-B and mutants in which His-29 or His-80 have been replaced by alanine (H29A, H80A) has been described elsewhere (Boehlein et al., 1994a). PCR cassette mutagenesis was used to replace Histidine-47 with alanine (H47A) or asparagine (H47N) using oligonucleotide primer pairs ss350R and ss65 or ss348R and ss65, respectively (Table 2-1) and pETB as a template. The PCR parameters involved 35 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for two minutes and the final cycle was followed by a 5 minute extension step at 72°C. The plasmid, pETB, and the PCR products were digested with HpaI and XhoI and the 50 bp product was cloned into pETB. The resulting plasmids were sequenced through the insertion and no alterations other than those intended were found. All wild-type and mutant proteins were expressed, and purified using standard procedures (Boehlein et al., 1994a). Protein concentrations were determined by the method of Bradford (1976) using an assay kit supplied by Bio-Rad and mouse immunoglobulin G to construct a standard curve. During the course of these investigations an alternate method of determining the protein concentration revealed that the current use of mouse immunoglobulin G as a standard gives erroneously high protein concentrations for AS-B. Amino acid composition analysis of AS-B, carried out by the Protein Chemistry Core Facility of ICBR at the University of Florida, showed that the actual value for the concentration of this enzyme is 2.7 times less than that obtained using the Bradford assay. Therefore, all of the values of enzyme

concentration and therefore all k_{cat} and k_{cat}/K_m values given in this chapter have been corrected by dividing the protein concentration by the factor 2.7.

TABLE 2.1
Oligonucleotides used in construction of site-directed mutants of AS-B

Oligo Number	Oligonucleotide Sequence
ss65	5' A GCT TCC CAT ATG TGT TCA ATT TTT GGC GTA TTC GAT 3'
ss350R	5' CGC CCC CGC GTT AAC GTC GAC AAT TGA CAG ACG TTC GGC GGC GAG A 3'
ss349R	5' CGC CCC CGC GTT AAC GTC GAC AAT TGA CAG ACG TTC GGC GGC GAG A 3'

Glutaminase Assays

Initial rates of L-glutamine and L-glutamic-γ-monohydroxamate (LGH) hydrolysis were determined by a modified procedure which uses glutamate dehydrogenase in the presence of NAD^+ to measure glutamate concentration (Bernt and Bergmeyer, 1974). Reactions (100 µl) containing 100mM Bis-Tris and Tris-HCl (pH 6.0-9.0) each (the kinetic constants appear to be independent of the nature and concentrations of these buffers), 8mM $MgCl_2$ and various concentrations of glutamine or LGH were initiated by the addition of wild-type (0.7-2 µg) or mutant (4 µg) AS-B and incubated at 37°C. A control containing denatured enzyme was used to monitor any glutamate formed from spontaneous breakdown. The reactions were terminated by boiling and a coupling reagent (300 mM glycine, 250

mM hydrazine, pH 9, 1 mM ADP, 1.6 mM NAD^+ , 2.2 units of glutamate dehydrogenase) was added and incubated 10 minutes at room temperature. The absorbance at 340 nm was measured and the concentration of glutamate was determined by comparison to a standard curve. Initial velocities were measured at 9 different substrate concentrations and each velocity was an average of 2-4 measurements. Stability of the wild-type and mutant AS-B enzymes at each pH was confirmed by incubation in buffer of the appropriate pH for 20 minutes followed by an assay of activity at pH 8. Initial velocity conditions were confirmed at each pH by linear plots of v versus time and v versus enzyme concentration.

Data Analysis

The values of K_m and k_{cat} were determined from least-squares fit to v_o vs [S] plots using the Prism software package supplied by Graphpad, Inc., (San Diego, CA) according to equation 1:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

Where v is the maximal velocity, [S] is the concentration of st V_{max} is the maximal velocity, and K_m is the Michaelis constant. The pH dependence of k_{cat}/K_m obtained from equation 1 were fit to the bell-shaped curve described by equation 2:

$$Y = \frac{Y_{lim}}{1 + 10^{(pk1-pH)} + 10^{(pH-pK2)}} \qquad (2)$$

In the above equation, Y is the observed k_{cat}/K_m at a given pH, Y_{lim} is the maximum value of k_{cat}/K_m, and pK_1 and pK_2 are the lower and higher acid dissociation constants, respectively.

Results

Effect of pH on the Steady-state Kinetic Parameters of wild-type AS-B Catalyzed Glutaminase

The steady-state kinetic parameters, k_{cat} and k_{cat}/K_m, for glutamine hydrolysis were measured as a function of pH. As illustrated in Figures 2.2 and 2.3, and Table 2.2, k_{cat} is constant over the pH range 6.0-9.0 whereas the k_{cat}/K_m exhibits a bell shaped curve. Since the pKa of the α-amino group of glutamine is 9.13, the K_m and thus k_{cat}/K_m were corrected for protonated glutamine using the Henderson-Hasselbach equation in an attempt to show how the ionization of the substrate might affect the k_{cat}/K_m-pH profile. Indeed, the correction caused a complete disappearance of the basic limb in the k_{cat}/K_m(app) plot (Figure 2.3). Non-linear regression of the experimental data to equation 1 allowed determination of the pKa for the non-corrected data which is shown in Table 2.5.

A comparison of the pH dependencies of the hydrolyses of glutamine and the alternate substrate, LGH, can provide information about whether these reactions follow the same mechanism. Therefore, a pH profile of the wild-type AS-B catalyzed LGH hydrolysis was also examined (figure 2.4 and 2.5, and Table 2.3). Similar to the glutamine profile, the k_{cat} appeared independent of pH for most of the pH range studied with slight increases at the low and high pH. The k_{cat}/K_m(app) profile for LGH is bell shaped with a peak

at neutral pH and an acidic and basic pKa close to that obtained with glutamine (Table 2.5). Unlike the glutamine profile, correction for protonated LGH had little effect on the pH-k_{cat}/K_m profile (Figure 2.5).

Kinetic Characterization of the Glutaminase Reaction catalyzed by the AS-B mutant H47N

As part of a continuing search for a histidine which may serve in a general acid-base capacity during AS-B catalyzed glutamine hydrolysis, Histidine-47 was examined using site-directed mutagenesis. Mutations were made which replaced the histidine with an alanine (H47A) or an asparagine (H47N) and the resulting enzymes were expressed. H47A was insoluble whereas H47N expressed at high levels and was successfully purified. The glutaminase activity of H47N was approximately 10-fold slower than that of wild-type AS-B (compare Tables 2.2 and 2.4). In addition to these changes in k_{cat}, there was a slight increase in the K_m value for glutamine.

pH Dependence of the Kinetic Constants of wild-type AS-B and its H47N

The pH dependence of the kinetic constants for H47N catalyzed glutamine hydrolysis was examined. The pH dependence of k_{cat} was minimal (figure 2.6) except for a slight decrease at low pH which was not evident in the wild-type. The pH profile of k_{cat}/K_m(app) revealed a bell-shaped curve (figure 2.7) similar to that of wild-type catalyzed LGH hydrolysis except the curve was slightly narrower. The decrease in k_{cat}/K_m(app) on the basic limb was evident even after the correction for protonated glutamine.

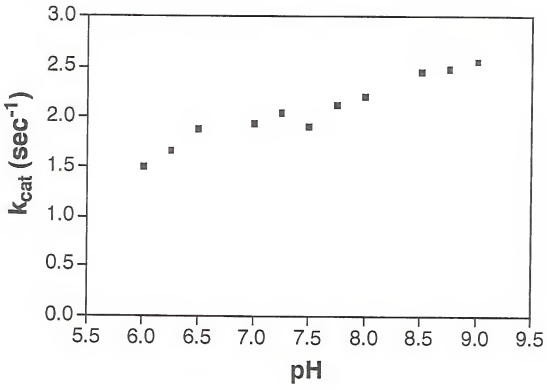


Figure 2.2 The pH dependence of k_{cat} for wild-type AS-B catalyzed glutamine hydrolysis. The kinetic constants were determined as described in materials and methods.

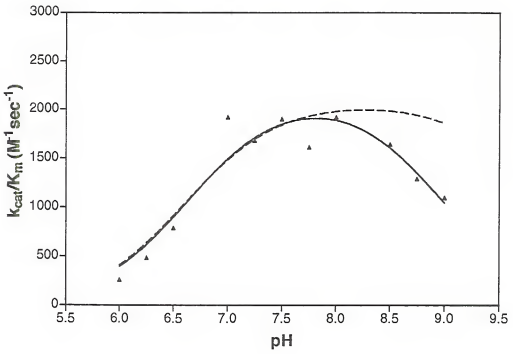


Figure 2.3 The pH dependence of k_{cat}/K_m for wild-type AS-B catalyzed glutamine hydrolysis. The kinetic constants were determined as described in the materials and methods. The solid line (—) represents the actual data. The dashed line (---) represents the data corrected for the protonation of glutamine.

Table 2.2
Kinetic constants of the AS-B-catalyzed hydrolysis of L-Glutamine at 37°C

pH	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}sec^{-1}$)
6.00	1.51 ± 0.05	5.78 ± 0.60	261
6.25	1.67 ± 0.10	3.44 ± 0.33	485
6.50	1.89 ± 0.03	2.4 ± 0.1	787
7.00	1.94 ± 0.03	1.01 ± 0.06	1921
7.25	2.05 ± 0.05	1.22 ± 0.08	1680
7.50	1.92 ± 0.03	1.01 ± 0.06	1901
7.75	2.13 ± 0.03	1.32 ± 0.07	1614
8.00	2.21 ± 0.03	1.15 ± 0.05	1922
8.50	2.46 ± 0.05	1.50 ± 0.06	1640
8.75	2.48 ± 0.05	1.92 ± 0.09	1292
9.00	2.56 ± 0.05	2.33 ± 0.12	1099

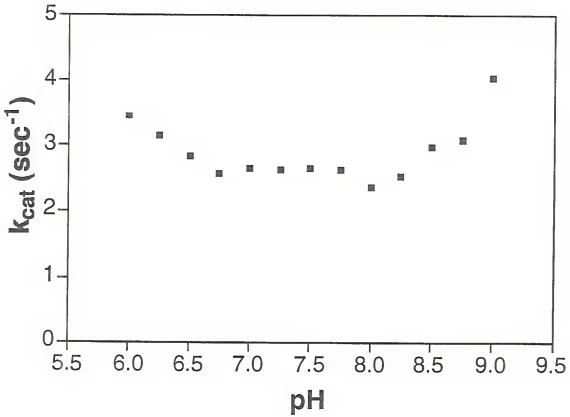


Figure 2.4 The pH dependence of k_{cat} for wild-type catalyzed LGH hydrolysis. The kinetic constants were determined as described in materials and methods.

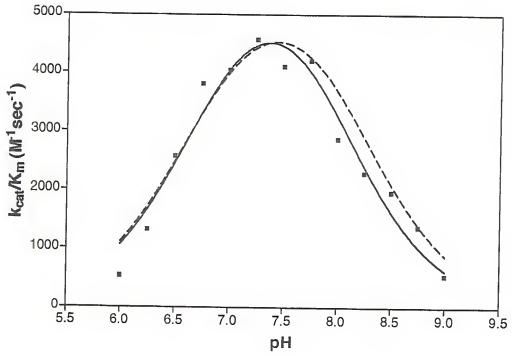


Figure 2.5 The pH dependence of k_{cat}/K_m for wild-type AS-B catalyzed LGH hydrolysis. The kinetic constants were determined as described in materials and methods. The solid line (—) represents the actual data. The dashed line (---) represents the data corrected for the protonation of glutamine.

Table 2.3:
Kinetic constants of the AS-B-catalyzed hydrolysis of L-glutamic-γ-monohydroxamate at 37°C

pH	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}sec^{-1}$)
6.00	3.46 ± 0.05	6.51 ± 0.29	531
6.25	3.16 ± 0.05	2.39 ± 0.15	1322
6.50	2.86 ± 0.03	1.11 ± 0.05	2577
6.75	2.59 ± 0.03	0.68 ± 0.02	3809
7.00	2.67 ± 0.03	0.66 ± 0.02	4045
7.25	2.65 ± 0.03	0.58 ± 0.03	4569
7.50	2.67 ± 0.03	0.65 ± 0.02	4108
7.75	2.65 ± 0.03	0.63 ± 0.03	4206
8.00	2.38 ± 0.05	0.83 ± 0.05	2867
8.25	2.54 ± 0.03	1.11 ± 0.05	2288
8.50	3.00 ± 0.05	1.53 ± 0.08	1961
8.75	3.10 ± 0.05	2.26 ± 0.11	1372
9.00	4.05 ± 0.19	7.41 ± 0.78	546

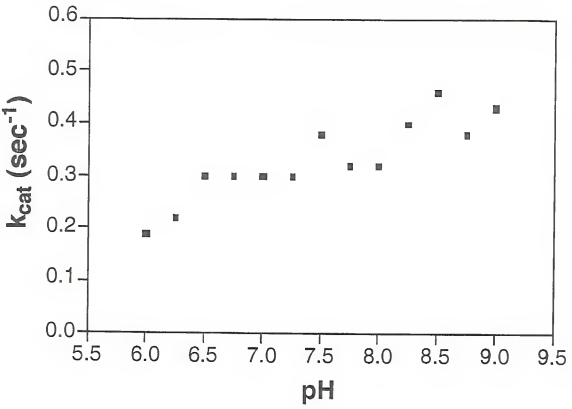


Figure 2.6 The pH dependence of k_{cat} for glutamine hydrolysis catalyzed by the AS-B mutant H47N. The kinetic constants were determined as described in materials and methods.

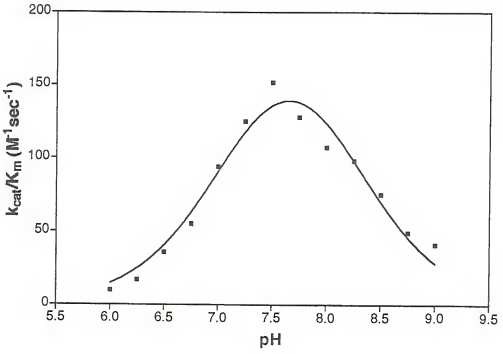


Figure 2.7 The pH dependence of k_{cat}/K_m for glutamine hydrolysis catalyzed by the AS-B mutant H47N. The kinetic constants were determined as described in materials and methods.

Table 2.4:
Kinetic constants for glutamine hydrolysis catalyzed by the AS-B mutant H47N at 37°C

pH	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}sec^{-1}$)
6.00	0.19 ± 0.01	18.6 ± 2.8	10
6.25	0.22 ± 0.008	13.1 ± 2.0	17
6.50	0.30 ± 0.005	8.3 ± 0.9	36
6.75	0.30 ± 0.008	5.4 ± 0.4	55
7.00	0.30 ± 0.005	3.2 ± 0.2	94
7.25	0.30 ± 0.003	2.4 ± 0.1	125
7.50	0.38 ± 0.005	2.5 ± 0.1	152
7.75	0.32 ± 0.003	2.5 ± 0.1	128
8.00	0.32 ± 0.003	3.0 ± 0.1	107
8.25	0.40 ± 0.005	4.1 ± 0.2	98
8.50	0.46 ± 0.008	6.1 ± 0.3	75
8.75	0.38 ± 0.01	7.8 ± 0.7	49
9.00	0.43 ± 0.01	10.5 ± 1.1	41

Table 2.5:
Values of pK_a for the k_{cat}/K_m-pH profiles for glutamine and LGH hydrolysis catalyzed by wild-type AS-B and for glutamine hydrolysis catalyzed by the AS-B mutant H47N

Enzyme	Substrate	k_{cat}/K_m	
		pK_1	pK_2
wild type	glutamine	6.7 ± 0.1	8.8 ± 0.1
	LGH	6.7 ± 0.1	8.0 ± 0.1
H47N	glutamine	7.2 ± 0.2	8.1 ± 0.2

TABLE 2.6:

Kinetic parameters for wild-type AS-B and the H29A, H80A, H47N, and R30A mutants at three values of pH

	pH 6.5			pH 7.5			pH 9.0		
	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})
wt	1.89 ± 0.03	2.40 ± 0.10	787	1.92 ± 0.03	1.01 ± 0.06	1901	2.56 ± 0.05	2.33 ± 0.12	1099
H29A	0.94 ± 0.10	34.47 ± 5.23	27	1.08 ± 0.13	10.36 ± 1.84	104	2.29 ± 0.57	115 ± 28	20
H80A	1.57 ± 0.11	30.92 ± 2.59	51	4.54 ± 0.65	5.02 ± 0.78	904	5.89 ± 0.54	58 ± 6	176
H47N	0.30 ± 0.005	8.3 ± 0.9	36	0.38 ± 0.005	2.46 ± 0.11	154	0.43 ± 0.01	10.47 ± 1.15	41

Comparison of the Kinetic Parameters of Wild-type, H80A, and H29A, at various pH values

The kinetic constants for the glutaminase activity of the three mutants (H29A, H47N, and H80A) are compared with wild-type in Table 2.6. Substitution of an alanine for the histidines at positions 29 and 80 has little effect on the k_{cat} at pH 6.5 and 7.5 while this substitution decreases k_{cat}/K_m significantly. However, unlike wild type the k_{cat} value of H80A increases with increasing pH to a value at pH 9 which is double that of wild type. The most striking effect caused by the mutations at positions 29 and 80 is seen in the dramatic increase in K_m especially at high pH.

Discussion

The importance of a histidine in the mechanism of glutamine hydrolysis catalyzed by AS-B and thus the mechanistic similarity between the AS-B catalyzed glutaminase reaction and the papain catalyzed peptide hydrolysis have been questioned for several years. Papain catalyzes the hydrolysis of peptide, amide, and ester bonds by the mechanism shown in (1). All substrates tested with papain have shown a bell-shaped pH dependence for k_{cat}/K_m with pK_a's of approximately 4 and 8.5. As shown in the simplified model below, it is considered that these ionizations represent the thiol of cysteine-25 and the imidazole of histidine-159 which are involved in the acylation process (Menard et al., 1990):

$$\text{-SH } {}^{+}\text{HIm-} \overset{pK_1\ 4}{\rightleftharpoons} \underset{\text{active enzyme form}}{\text{-S}^{-}\ {}^{+}\text{HIm-}} \overset{pK_2\ 8.5}{\rightleftharpoons} \text{-S}^{-}\ \text{Im-}$$

In order to understand how acid-base catalysis occurs in the AS-B active site during glutamine hydrolysis, the pH dependence of the kinetic constants has been examined for this enzyme. For both glutamine and LGH, the k_{cat}/K_m value has a bell-shaped pH dependency. In contrast, k_{cat} appears independent of pH in the range of 6-9. The similarity in pH profiles for these two substrates suggests that their hydrolysis occurs by similar mechanisms. Furthermore, the values of k_{cat}/K_m for LGH are about 2-fold higher than those for glutamine (compare Tables 2.2 and 2.3). If substrate hydrolysis follows a mechanism like that of papain, then N-protonation of the leaving group on the first tetrahedral intermediate is required for C-N bond cleavage to occur. Therefore, the faster acylation rate for LGH, reflected in k_{cat}/K_m, is consistent with the fact that hydroxylamine ($pK_a \approx 8.2$) is a better leaving group than ammonia ($pK_a \approx 9.2$). The pH independence of k_{cat} may arise from a change in rate determining step between two steps with similar rates. However, further investigation, using a more extensive pH range is necessary for a clearer picture of the ionizations affecting k_{cat}. Unfortunately, the AS is highly unstable when the pH is greater than 9 or less than 6.

The bell-shaped curves displayed in the k_{cat}/K_m profiles for AS-B hydrolysis of glutamine and LGH (with pK_a values of 6.6 and 8.3) are narrower than that reported for papain. The stability of the ion-

pair in papain is reflected in the width of the pH-profile for k_{cat}/K_m (Menard et al., 1991). Therefore, it is possible that AS-B contains an ion pair like that of papain but lacks some of the factors which play a role in ion pair stabilization. On the other hand, the pH profile of k_{cat}/K_m may relate to ionizations in free substrate. In this study, the pK_a values of the α-amino groups of the substrates are 9.13 therefore, the possibility that the alkaline pK_a values observed in the k_{cat}/K_m profiles arise from the ionization of glutamine and LGH cannot be excluded. In fact, the basic limb of the glutamine k_{cat}/K_m profile is completely eliminated when K_m values are corrected for protonated glutamine. However, the same treatment has little effect on the k_{cat}/K_m profile for LGH.

While the above examination of the kinetic behavior of wild-type AS-B as a function of pH indicates the potentiality of an ion pair similar to that of papain, past efforts have failed to reveal a histidine which functions as part of that pair. In this study, substituting an asparagine for histidine at position 47 resulted in a reduction of both k_{cat} and k_{cat}/K_m of approximately 10-fold for the glutaminase activity. While these changes are significant, a much more dramatic effect was expected if this residue were playing such an essential role in catalysis as has been postulated. For example, replacement of histidine-353 with an asparagine in the class I enzyme, CPS, resulted in a drop in glutaminase activity from 1.1 µmol/h•mg for wt to ≤0.006 µmol/h•mg for H353N (Miran et al, 1991). Subsequent structural studies which showed that this residue was in position to interact with the active site histidine added further support that His-353 was involved in acid-base catalysis (Thoden et al., 1997).

Additionally, the pH profiles for glutamine hydrolysis catalyzed by H47N (Figures 2.6 & 2.7) resemble those of wt including similar pK_a values of 7.2 and 8.5 (Table 2.5) which suggests that histidine-47 is not responsible for one of these ionizations.

Previous investigations of H29 and H80 failed to reveal that a histidine in either of these positions was essential for AS-B catalyzed asparagine synthesis (Boehlein et al., 1994a). While these studies were performed at pH 8 in the presence of an imidizole buffer, it is possible that the importance of the active site histidine could have been masked. Table 2.6 presents the results of a further investigation using the glutaminase activity at pH 6.5, 7.5 and 9.0. The lack of a significant decrease in the k_{cat} value for the glutaminase reaction catalyzed by these mutants confirms the previous conclusions that these histidines probably are not involved as part of the essential cys-his dyad which has been proposed.

Interpretations of this and any pH analysis of an enzymatic reaction rely on a number of assumptions which have been summarized by Knowles (1976). First, as noted by Peller and Alberty (1959), the association of the pK_a values of the pH-k_{cat}/K_m with ionizations of the free enzyme and free substrate assumes that only one ionization state of the enzyme is directly convertible to product. In fact, the pH dependence of the k_{cat}/K_m values observed in papain catalyzed substrate hydrolysis has been found to be more complex than originally thought (Brocklehurst, 1994). Several studies have provided evidence that the development of the full nucleophilic character of the ion pair depends not only on the ionization at pH 4 but on a second ionization at pH 4 and, in some cases, ionization with

pK_a 5 (Brocklehurst, 1988 a,b). Altered pK_a values also may result if the rate constant for substrate dissociation is less than the net rate constant for product formation. This is unlikely to be the case for AS-B glutamine hydrolysis since investigations into the dissociation constant of glutamine have suggested that a rapid equilibrium mechanism is plausible (see chapter 4). Determination of the pK_a of a competitive inhibitor or of the backwards reaction will be necessary to confirm this assumption.

With respect to these assumptions it is important to take heed in comparison of the pH profiles of AS-B and papain. On the other hand, with the results presented here, it is difficult to envision a role for histidine-47 in acid-base catalysis when one considers the lack of effect its replacement with asparagine made on the pH profiles. In light of the presence of this histidine in many asparagine synthetases as well as other class II amidotransferases it was important to include this residue among potential acid-base catalysts. Indeed, the 10-fold loss of activity in the H47N mutant may reflect a minor or indirect importance of this residue in glutamine binding and/or utilization. On the other hand, the importance of the histidine to papain activity is underscored in the complete conservation of this amino acid across the entire family of cysteine proteases. The lack of such a completely conserved histidine in the class II family of amidotransferases suggests that an alternate mechanism of acid-base catalysis probably occurs in these enzymes and therefore demonstrates dissimilarities in mechanism for the two families of enzymes.

CHAPTER 3
EFFECT OF TEMPERATURE ON GLUTAMINE HYDROLYSIS CATALYZED BY ASPARAGINE SYNTHETASE B

Introduction

The following chapters present experiments which are aimed at the determination of rate constants and the identification of intermediates in order to develop a descriptive model of the glutaminase reaction. Much of the investigation requires a very slow reaction rate and, therefore, the reactions were examined at 5°C. Variation in temperature can cause alterations in the mechanism of a reaction including differences in rate determining steps. In order to validate the comparison of the results from experiments at 5°C with those performed at the temperature at which the reaction is normally investigated (37°C), the steady state kinetics of glutamine hydrolysis will be examined over a range of temperatures and Arrhenius plots will be used to detect any changes in rate limiting step with temperature for this reaction. The temperature dependence of LGH hydrolysis catalyzed by wild-type AS-B and that of glutamine hydrolysis catalyzed by the AS-B mutant R30A will be examined in a similar manner and the activation energies of the three reactions will be compared. Similarity in the pH profiles for glutamine and LGH hydrolysis presented in the previous chapter suggests that these reactions occur by a common mechanism. Further evidence for a common mechanism could be obtained

through similarities in the activation parameters which would suggest that the same step is rate limiting in both reactions. In contrast, work in the following chapters suggests that glutamine hydrolysis catalyzed by the AS-B mutant, R30A, has a different rate limiting step than that catalyzed by wt AS-B and these differences might be revealed in different values for the activation parameters.

A complete description of the glutaminase reaction requires 1) the order of product release, 2) the inhibition constants of the products, and 3) knowledge of whether or not the reaction is reversible. Therefore, in order to address these issues, product inhibition studies were employed and the ability of the enzyme to catalyze the synthesis of glutamine and LGH was examined.

Materials and Methods

Chemicals and Reagents

[^{14}C]-glutamine was purchased from Amersham. Scintillation fluid, ScintiVers™ II* was obtained from Fischer (Orlando, FL). DE-81 anion exchange chromatography paper was purchased from Whatman (Hillsboro, OR). Other chemicals, including L-glutamine and L-glutamic-γ-monohydroxamate, and hydroxylamine were obtained from SIGMA Chemical Company (St. Louis, MO) and were of the highest commercial purity.

Enzyme Preparation

Construction of recombinant wild-type AS-B and the mutant AS-B in which R30 has been replaced by alanine (R30A) have been described elsewhere (Boehlein et al., 1994a,b). Both wild-type and mutant proteins were expressed, and purified using standard

procedures (Boehlein et al., 1994a). Protein concentrations were determined by the method of Bradford (1976) using an assay kit supplied by Bio-Rad and mouse immunoglobulin G to construct a standard curve. During the course of these investigations an alternate method of determining the protein concentration revealed that the current use of mouse immunoglobulin G as a standard gives erroneously high protein concentrations for AS-B. Amino acid composition analysis of AS-B, carried out by the Protein Chemistry Core Facility of ICBR at the University of Florida, showed that the actual value for the concentration of this enzyme is 2.7 times smaller than that obtained using the Bradford assay. Therefore, all of the values of enzyme concentration and therefore all k_{cat} and k_{cat}/K_m values given in this chapter have been corrected by dividing the protein concentration by the factor 2.7.

Glutaminase Assays

Initial rates of L-glutamine and L-glutamic-γ-monohydroxamate (LGH) hydrolysis were determined by a modified procedure which uses glutamate dehydrogenase in the presence of NAD^+ to measure glutamate concentration (Bernt & Bergmeyer, 1974). Reactions (100 µl) containing 100mM Bis-Tris and Tris-HCl (pH 8.0) each, 8mM $MgCl_2$ and various concentrations of glutamine or LGH were initiated by the addition of wild-type or mutant R30A AS-B and incubated at various temperatures (4°C-40°C). A control reaction lacking enzyme was used to monitor any glutamate formed from spontaneous breakdown. The reactions were terminated by the addition of 20 µl of 1N acetic acid. A coupling reagent (300 mM glycine, 250 mM hydrazine, pH 9. 1 mM ADP, 1.6 mM NAD^+ , 2.2 units of glutamate dehydrogenase) was added

and incubated 10 minutes at room temperature. The absorbance at 340 nm was measured and the concentration of glutamate was determined by comparison to a standard curve. Initial velocities were measured at 9 different substrate concentrations and each velocity was an average of 3 measurements. Stability of the wild-type and mutant AS-B enzymes at each temperature was confirmed by incubation in buffer of the appropriate temperature for various amounts of time followed by an assay of activity at pH 8. Initial velocity conditions were confirmed at each pH by linear plots of v versus time and v versus enzyme concentration. The pH of the buffer was adjusted to pH 8 at each temperature studied to account for the pKa change with temperature.

Determination of K_m for glutamate and hydroxylamine in the AS-B catalyzed synthesis of LGH

The Michaelis constant, K_m, of glutamate in the synthesis of LGH was determined by incubating wild-type AS-B with various concentrations of glutamate and a saturating concentration of hydroxylamine (150 mM) in Tris-HCl, pH 8.0, at 37°C for 15 min. In a similar manner the K_m for hydroxylamine was determined by incubating the wild-type enzyme with various concentrations of hydroxylamine and a saturating concentrations of glutamate (150 mM) in Tris-HCl, pH 8.0, at 37°C for 20 min. In both cases, the 300 µl reaction was terminated by the addition of 100 µl of 16 % TCA. LGH formation was determined in a final volume of 500 µl by adding a solution containing 80% TCA, 6N HCl, and 10% $FeCl_3$ in 0.02 N HCl to the remaining reaction, centrifuging the samples in a microcentrifuge to remove particulates, and measuring the absorbance of the

hydroxamate-$FeCl_3$ complex at 540 nm. A standard curve using a stock solution of authentic LGH was used to quantitate the product formed in these reactions.

The ability of AS-B to catalyze the synthesis of glutamine also was examined. Wild-type AS-B (37 μg) was combined with glutamate and ammonium chloride (100 mM each) in Tris-HCl (200 mM) in a total volume of 50 μl. The reactions were incubated for 4 hours at 37°C followed by the addition of 20 μl of 2N acetic acid. The terminated reactions were filtered through a 2 μm filter and derivatized with phenylisothiocyanate (PITC). The glutamine and glutamate derivatives were separated by HPLC and quantitated spectroscopically. This assay procedure can detect less than 100 pmoles under our standard procedures

Product inhibition of the glutaminase reaction

Wild-type AS-B was incubated in a 100 μl reaction containing various concentrations of [^{14}C] glutamine (SA 880-20,000 dpm/nmol) in 100mM Bis-Tris and Tris-HCl at 37°C for 10 min in the presence of various concentrations of glutamate or NH_4Cl. The reactions were terminated by the addition of 10 μl of 1N acetic acid. Glutamate was separated from glutamine on Whatman DE-81 ion-exchange chromatography paper using deionized distilled water as the mobile phase. After 6 hours, elution was stopped, and the paper was dried and cut into two strips to separate glutamine from glutamate. The radioactivity associated with each strip was counted in ScintiVersII* scintillation fluid on a Beckman LS 61C scintillation counter. Control experiments were carried out in an identical manner except that the enzyme was excluded from the reaction mixture. The separation of

the amino acids was visualized in a non-radioactive control by spraying the paper with 0.2% ninhydrin in 95% ethanol. Glutamine was found to migrate with the solvent front while glutamate remained at the origin. The amount of ^{14}C-labeled glutamate formed in the reaction was determined by subtracting the percentage of total radioactive counts located at the origin in the control reactions from the percentage in each sample.

In a similar manner, the inhibition of glutamate also was examined at 5°C except that the reaction time was 40 min.

Data Analysis

The values of K_m and k_{cat} were determined from least-squares fit to v_o vs [S] plots using the Prism software package supplied by Graphpad, Inc., (San Diego, CA) according to equation 1:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

Where v is the initial velocity, [S] is the concentration of substrate, V_{max} is the maximal velocity, and K_m is the Michaelis constant.

As shown in the equation below, the value for $-E_a/RT$ can be derived from the slope of a plot of the log k_{cat} versus 1/T (K) where A is a constant which is related to the collision frequency and steric factors.

$$k = Ae(-E_a/RT) \qquad (2)$$

Values for the energy, enthalpy, and entropy of activation then were calculated using the following equations:

$$\Delta H^* = E_a - RT \qquad (3)$$

$$\Delta G^* = RT\ ln(k_B T/h) - RTln(k) \qquad (4)$$

$$\Delta G^{*} = \Delta H^{*} - T\Delta S^{*} \qquad (5)$$

where ΔH^{*} is the enthalpy of activation, R is the gas constant, k_B is boltzmans constant, h is Planck's constant, k is the forward rate constant; ΔG^{*} is the free energy of activation, and ΔS^{*} is the entropy of activation.

Results

Effect of temperature on glutamine and LGH hydrolysis catalyzed by wild-type AS-B and the AS-B mutant R30A

The steady-state kinetic parameters of glutamine and LGH hydrolysis were determined at a range of temperatures from 5° C to 40° C. The Arrhenius plots (Figures 3.1 & 3.2) were observed to be linear indicating either that there was no change in the rate-determining step over the temperature range studied or that the activation energies for the limiting steps were very similar.

The temperature dependence of the catalytic rate constants of the R30A-catalyzed hydrolysis of glutamine also displayed linearity in the Arrhenius plot, as shown in Figure 3.3. The activation parameters are given in Table 3.1.

LGH synthesis catalyzed by wild-type AS-B

AS-B was found to catalyze the formation of LGH from glutamate and hydroxylamine. The reaction rate of 0.29 sec^{-1} (Table 3.2, Figures 3.4 & 3.5) was about 12% of that of the corresponding forward rate of 2.4 sec^{-1} at pH 8 (see chapter 2, Table 2.3), and was fast enough to obtain the kinetic parameters of LGH synthesis catalyzed by wild-type AS-B (Table 3.2).

The steady-state kinetic parameters of reversible reactions can

be used to calculate the equilibrium constant for the overall reaction using the Haldane relationship for a one-substrate/two-product reaction given in equation (6) (Cleland, 1963):

$$K_{eq} = \frac{V_f K_{ip} K_q}{V_r K_{ia}} = \frac{V_f K_p K_{iq}}{V_r K_a} \qquad (6)$$

where K_{eq} is the equilibrium constant for the conversion of substrate to product, V_f and V_r represent the forward and reverse reaction rates, respectively, and the indices p, q, and a refer to hydroxylamine, glutamate and LGH, respectively. Using equation (6) and the following parameters, obtained at 37°C: k^f_{cat} = 2.4 sec^{-1}, $K_{m(NH2OH)}$= 95 mM, $K_{i(glu)}$=182 mM, k^r_{cat}= 0.29 sec^{-1}, $K_{m(LGH)}$= 0.83 mM; a value of K_{eq}=172 was calculated. Using this value for K_{eq} and the equation, $\Delta G^o = -RT\ln K_{eq}$, the difference in free energy between substrates and products, ΔG^o, was determined to be -13.2 kJ/mol K.

Product inhibition glutamine hydrolysis catalyzed by wild-type AS-B

Figures 3.6 and 3.7 show the plots for the inhibition of the glutaminase reaction by glutamate at 5°C and 37°C, respectively. The K_i values for glutamate determined from the replot of the slope versus glutamate concentration (Figure 3.8 & 3.9) at 5°C and 37°C were 260 mM and 182 mM, respectively. An attempt to obtain the K_i for ammonia was made. However, NH_4Cl concentrations up to 200 mM seemed to have little effect on the rate of the glutaminase reaction.

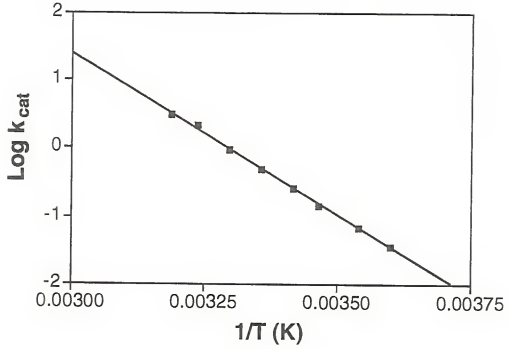


Figure 3.1: Temperature dependence of glutamine hydrolysis catalyzed by wild-type AS-B. The initial rates were determined at pH 8.0 in 100 mM Bis-Tris and Tris-HCl as described in the materials and methods.

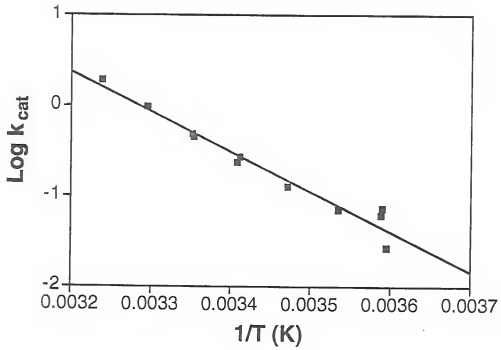


Figure 3.2: Temperature dependence of LGH hydrolysis catalyzed by wild-type AS-B. The initial rates at each temperature were determined at pH8.0 in 100 mM Bis-Tris and Tris-HCl as described in materials and methods.

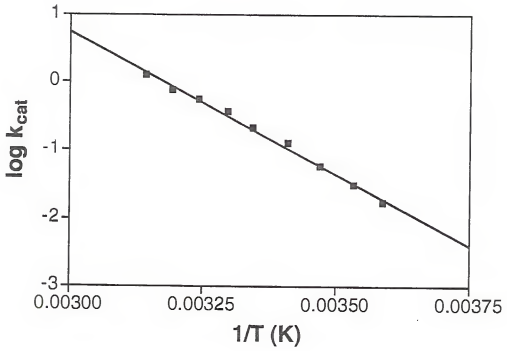


Figure 3.3: Temperature dependence of glutamine hydrolysis catalyzed by the AS-B mutant R30A. The initial rates at each temperature were determined at pH 8.0 in 100 mM Bis-Tris and Tris-HCl as described in materials and methods.

Table 3.1: Thermodynamic properties of glutamine and LGH hydrolysis catalyzed by wild-type AS-B and the R30A mutant.

Substrate/ enzyme	$\Delta G^{\ddagger}$ kJ mol^{-1} (37°C)	$\Delta H^{\ddagger}$ kJ mol^{-1} (37°C)	$\Delta S^{\ddagger}$ J $mol^{-1}K^{-1}$ (5-40°C)	$T\Delta S^{\ddagger}$ kJ mol^{-1} (37°C)
gln/wt	167	89	-252	-78
LGH/wt	152	82	-226	-70
gln/R30A	147	77	-226	-70

The initial rates were determined at pH 8.0 in 100 mM Bis-Tris and Tris-HCl at temperatures ranging from 5-40°C, under conditions described in the materials and methods.

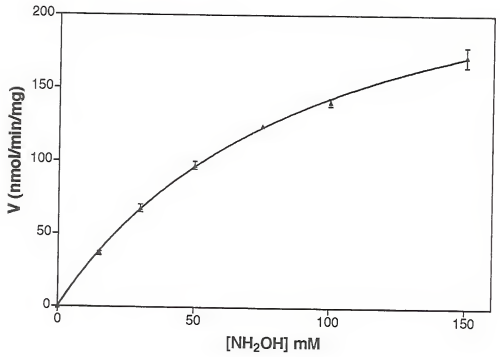


Figure 3.4: Effect of hydroxylamine concentration on the synthesis of LGH catalyzed by wild-type AS-B. Wild-type AS-B (18.5 μg) was incubated with various concentrations of hydroxylamine and saturating concentrations of glutamate (150 mM) in Tris-HCl, pH 8.0 at 37°C for 20 min. The reaction was terminated by the addition of 16% TCA and LGH formation was measured as described in materials and methods.

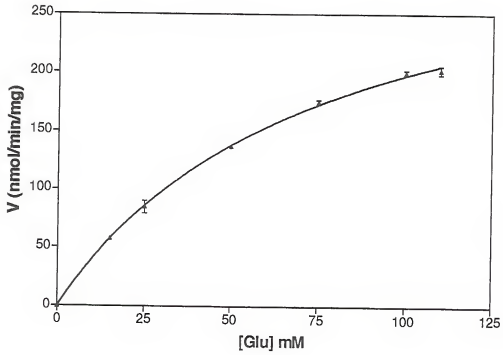


Figure 3.5: Effect of glutamate concentration on the synthesis of LGH catalyzed by wild-type AS-B. Wild-type AS-B (18.5 μg) was incubated with various concentrations of glutamate and a saturating concentration of hydroxylamine (150 mM) in Tris-HCl, pH 8.0 at 37°C for 15 min. The reactions were terminated by the addition of 16% TCA and the formation of LGH was measured as described in materials and methods.

Table 3.2: Kinetic constants for the synthesis of LGH catalyzed by wild-type AS-B.

Substrate	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})
glutamate	0.36 ± 0.02	76 ± 5	4.7
hydroxylamine	0.29 ± 0.01	95 ± 7	2.9

The initial rates were determined at pH 8.0 in 100 mM Bis-Tris and Tris-HCl at 37°C, by varying one substrate while keeping the second substrate saturating. The conditions of the reaction and the determination of the LGH formed in the reaction are described in the materials and methods.

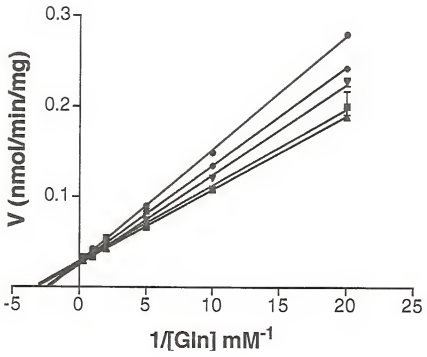


Figure 3.6: Glutamate inhibition of the glutaminase reaction catalyzed by wild-type AS-B at 5°C. The rate of glutamine hydrolysis was determined as described in the materials and methods in the presence of 0 (■), 10 (▲), 50 (▼), 100 (◆), and 136 (●) mM glutamate.

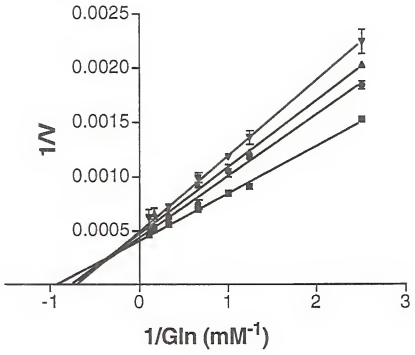


Figure 3.7: Glutamate inhibition of the glutaminase reaction catalyzed by wild-type AS-B at 37°C. The rate of glutamine hydrolysis was determined as described in the materials and methods in the presence of 0 (■), 40 (●), 80 (▲), and 100 (▼) mM glutamate.

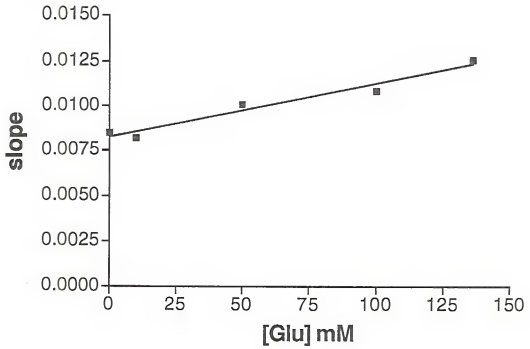


Figure 3.8: Replot of the slope from the double-reciprocal plots of glutamine hydrolysis at 5°C versus glutamate concentration.

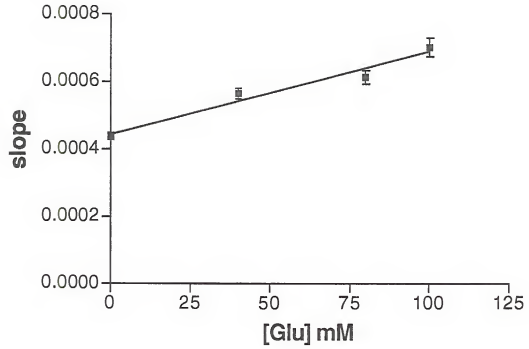


Figure 3.9: Replot of the slope from the double-reciprocal plots of glutamine hydrolysis at 37°C versus glutamate concentration.

Discussion

Many of the experiments in the following chapters required a reaction rate slower than that observed at 37°C, the standard temperature at which the reaction is examined. One way to obtain a slower rate is to examine the reaction at low temperatures therefore, many of the experiments in the following chapters have been conducted at 5°C. In preparing for these experiments it was important to know whether the results obtained at 5°C could be compared to those at the standard temperature. Temperature variations often cause changes in the rate-limiting step of a reaction. Since non-linear Arrhenius plots are indicative of such changes, the temperature dependence of the glutaminase reaction was examined to confirm that there was not a change in the rate limiting step between 5°C and 37°C. In the case of the glutaminase reaction, it is difficult to conclude that a single step is rate determining across the entire range of temperatures studied. Certainly, the linear Arrhenius plots suggest that a change in the rate limiting step between reactions at 5°C and 37°C is unlikely. However, it should be noted that two steps in the reaction may have similar activation energies, and therefore a change in rate limiting step would remain unnoticed in these experiments. Such an event is a possibility in AS-B catalyzed glutamine hydrolysis as demonstrated by the unexpected similarity in activation constants for reactions catalyzed by wild-type and the mutant R30A (Table 3.1 and Figures 3.1 and 3.3). Partitioning experiments in chapter 4 imply that in reactions catalyzed by R30A, hydrolysis of the thioester is rate limiting. In contrast, direct measurements of the deacylation step of glutamine hydrolysis suggest that an additional step contributes to the

overall rate of the reaction catalyzed by the wild-type enzyme. The differences in the nature of the rate determining step in the reactions catalyzed by the wild-type and mutant enzyme were expected to be reflected in the activation parameters. The lack of any differences in the parameters suggests that the two slow steps in the wild-type catalyzed reaction have similar activation energies.

The patterns of product inhibition can provide information on the order of product release. However, the studies of product inhibition presented here were inconclusive due to the inability of ammonia to inhibit the glutaminase reaction. On the other hand, the examination of glutamate inhibition provided a needed inhibition constant which will be used in the model described in chapter 7.

The inability of ammonia to inhibit the glutaminase reaction and the lack of detectable glutamine synthesis using ammonia and glutamate as substrates suggests that the acylation step is essentially irreversible under the conditions of the examination. With the ability to detect glutamine concentrations to 0.1 nmol (see materials and methods), an upper limit on the turnover number of $k_{cat} < 5x10^{-4}$ sec^{-1} can be set for glutamine synthesis at 37°C catalyzed by the wild-type AS-B under the conditions studied. In contrast, the reversibility of LGH hydrolysis catalyzed by wild-type AS-B was easily demonstrated and has a k_{cat} which is 12% of the forward reaction. The difference in the ability to synthesize the reverse reaction may be a reflection of the greater nucleophilicity of hydroxylamine. The kinetic parameters, k_{cat} and K_m, obtained from the analysis of LGH synthesis allowed the determination of the equilibrium constant for the overall reaction.

The free energy, ΔG°, derived from this equilibrium constant was calculated to be -13.2 kJ/mol.

CHAPTER 4
DETERMINATION OF THE RATE DETERMINING STEP IN ASPARAGINE SYNTHETASE B-CATALYZED GLUTAMINE HYDROLYSIS

Introduction

Asparagine synthetase B (AS-B) is thought to be organized into two functional domains: the glutamine amide transfer (GAT) domain, which catalyzes the hydrolysis of glutamine to glutamate; and the synthetase domain, which catalyzes the formation of a β-aspartyl-AMP intermediate and the synthesis of asparagine. While the overall reaction involves three substrates, four products, and possibly twenty rate constants; the ability of the enzyme to catalyze glutamine hydrolysis in the absence of the other substrates provides the opportunity to examine glutamine utilization under a simpler one substrate, two product reaction. A detailed description of this reaction would provide a useful tool in understanding the roles played by conserved amino acid residues of the GAT domain in glutamine utilization. Therefore, this chapter presents a first step towards characterizing the glutaminase reaction catalyzed by AS-B.

Analysis of the amino acid sequence of the GAT domain suggests that AS-B belongs to a family of enzymes known as the glutamine amidotransferases (Zalkin, 1993). Like AS-B, the enzymes of this family catalyze nitrogen transfer using glutamine as the primary source of nitrogen. While all enzymes in the glutamine amidotransferase family contain a cysteine which is essential for

glutamine dependent activity, the position of this cysteine as well as other sequence differences in the GAT domain further divides the family into two classes. The essential cysteine residue is located within the GAT domain for class I enzymes whereas it resides at the N-terminus in the class II enzymes such as glutamine phosphoribosylpyrophosphate amidotransferase (GPA) (Tso et al., 1982) , glucosamine-6-phosphate synthetase (GFAT) (Badet et al., 1987), and asparagine synthetase (Humbert et al., 1980). Initially, site directed mutagenesis studies suggested that the N-terminal cysteine as well as a histidine and aspartate residue were involved in the reaction catalyzed by GPA (Mei & Zalkin, 1989). As this catalytic triad is found in many thiol proteases (Brocklehurst et al., 1987), it was proposed that the glutamine hydrolysis reaction catalyzed by the class II enzymes occurs via a mechanism analogous to that of peptide hydrolysis catalyzed by the thiol proteases, such as papain. This hypothesis was extended to the class I enzymes as well (Mei & Zalkin, 1989). Since then, the importance of a catalytic triad composed of a conserved cysteine, histidine, and glutamate residues has been demonstrated for the class I enzymes through site-directed mutagenesis (Amuro et al., 1985; Miran et al., 1991) and x-ray crystallography (Tesmer et al., 1996; Thoden et al., 1997).

On the other hand, the picture remains more ambiguous for the class II amidotransferases as subsequent studies confirmed only the importance of the conserved N-terminal cysteine residue (Boehlein et al., 1994a). In fact, a more recent alignment of the GAT domain which includes a greater number of amidotransferase sequences reveals that there are no histidines which are conserved across this

entire class of enzymes (Boehlein et al., 1994a; Zalkin, 1993). Moreover, studies using site-directed mutagenesis of AS-B (Chapter 2; Boehlein et al., 1994a) and x-ray crystallography of GFAT (Isupov et al., 1996) and GPA (Kim et al., 1995) suggest that the class II amidotransferases function differently than papain with respect to acid-base catalysis. While the lack of a conserved histidine in the class II amidotransferases contrasts with the essentiality of such a residue in thiol proteases, recent evidence for an acyl enzyme intermediate (chapter 5 & 6) formed during glutamine hydrolysis catalyzed by AS-B provides a basis for comparison of the hydrolysis reactions catalyzed by the two enzyme families. A minimal description of amide hydrolysis catalyzed by papain, which occurs via a thioester intermediate (E-TE), is shown in equation 1 below:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} \underset{+P_1}{E\text{-}TE} \xrightarrow{k_3} E + P_2 \qquad \text{(equation 1)}$$

In relating this equation to glutamine hydrolysis catalyzed by AS-B, ES is the enzyme-substrate complex, E-TE is the acylenzyme intermediate, and P_1 and P_2 are ammonia and glutamate, respectively. In an effort to understand the mechanism of glutamine hydrolysis catalyzed by AS-B, the glutaminase reaction will be examined in order to develop a model for this particular aspect of the enzymatic reaction. The minimal model shown above will be used as a starting point in this investigation to provide evidence (in addition to that shown in chapter 5 for the existence of a thioester intermediate, to determine whether steps leading to its formation or

those involved in its breakdown are rate limiting, and to compare and contrast catalytic behavior of three groups of hydrolytic enzymes: a class II amidotransferase (AS-B), a class I amidotransferase (CAD), and a thiol protease (papain).

Materials and Methods

Chemicals and Reagents

^{14}C-glutamine was purchased from Amersham. Scintillation fluid, ScintiVers™ II* was obtained from Fischer (Orlando, FL). DE-81 anion exchange chromatography paper was purchased from Whatman (Hillsboro, OR). Other chemicals, including L-glutamine and L-glutamic-γ-monohydroxamate, hydroxylamine, and 6-diazo-5-oxonorleucine (DON) were obtained from SIGMA Chemical Company (St. Louis, MO) and were of the highest commercial purity.

Enzymes

The construction of recombinant AS-B and the AS-B mutant, R30A, has been described previously (Boehlein et al., 1994a, 1994b). The enzymes were obtained by overexpression and purified using standard procedures (Boehlein et al., 1994a). Protein concentration was determined by the method of Bradford (1976) using an assay kit supplied by Biorad and immunoglobulin G as a standard. Recent examination of the protein concentration using amino acid analysis of AS-B, performed by the Protein Core Facility of ICBR at the University of Florida, gave a value for protein concentration which was 2.7-fold lower than that obtained with the previous method. Therefore, in this report the enzyme concentration has been corrected by dividing the enzyme concentrations by the factor 2.7.

Steady State Kinetics

The kinetic parameters of AS-B-catalyzed glutamine, LGH, and L-glutamine-hydrazide hydrolysis were obtained in an end-point assay which uses glutamate dehydrogenase in the presence of NAD^+ to measure the concentration of glutamate formed (Bernt & Bergmeyer, 1974). Reactions (100 µl) containing 100mM Bis-Tris and Tris-HCl (pH 7.5) each, 8mM $MgCl_2$ and various concentrations of glutamine, LGH, or glutamic acid-γ-hydrazide were initiated by the addition of 1.5 µg wild-type AS-B and incubated at 37°C. After 10 minutes, the reactions were terminated by boiling for 2 minutes. Reactions in which the enzyme was added to the mix during the termination period of boiling were used to monitor any glutamate formed from spontaneous breakdown. A coupling reagent (300 mM glycine, 250 mM hydrazine, pH 9, 1 mM ADP, 1.6 mM NAD^+ , 2.2 units of glutamate dehydrogenase) was added to the reaction and the resulting mixture was incubated 10 minutes. The absorbance at 340 nm was measured and the concentration of glutamate was determined by comparison to a standard curve. Initial velocities were measured at 9 different substrate concentrations and each velocity was an average of 2-4 measurements.

The values of K_m and k_{cat} were determined from least-squares fit to v_o vs [S] plots using the Prism software package supplied by Graphpad, Inc., (San Diego, CA).

Determination of the K_i for glutamine and LGH

The rate of inactivation by the glutamine analog, 6-diazo-5-oxonorleucine (DON), was determined by incubating 30 µg wt AS-B in a 300 µl solution containing 100 mM Bis-Tris and Tris-HCl, pH 8 and

varying concentrations of DON at 5°C. At various time points 20 µl aliquots were removed from the incubation mixture and the residual activity was measured in 200 µl buffer containing 12 mM glutamine. Control experiments measuring the activity in the presence of diluted inhibitor concentrations verified that DON did not interfere in the assay of residual activity.

Glutamine protection of DON inhibition was examined as described above except that wt AS-B was incubated with varying concentrations of DON in the presence of either 20 µM or 50 µM glutamine. Alternatively, LGH protection of DON inactivation of the wild type enzyme and glutamine protection of R30A inactivation were examined by incubating wild-type or R30A AS-B with a constant concentration of DON (6 µM for wild type and 650 µM for R30A) and varying concentrations of LGH or glutamine, respectively.

An analysis of glutamate formation in AS-B catalyzed glutamine hydrolysis as a function of time.

Glutamine hydrolysis catalyzed by wt AS-B was examined at 5°C, a temperature at which the reactions were slow enough to mix by hand. The reactions were initiated by adding 0.37 or 0.74 nmol wt AS-B to a 50 µl solution containing 100 mM Bis-Tris and Tris-HCl (pH 8) and [^{14}C]-glutamine (2mM, SA = 9 nCi/µl). After the appropriate amount of time, the reactions were terminated with 20 µl of 2N acetic acid. Glutamate was separated from glutamine on Whatman DE-81 ion-exchange chromatography paper using deionized distilled water as the mobile phase. After 6 hours, elution was stopped, and the paper was dried and cut into two strips to separate glutamine from glutamate. The radioactivity associated

with each strip was counted in ScintiVersII* scintillation fluid on a Beckman LS 61C scintillation counter. Control experiments were carried out in an identical manner except that the enzyme was excluded from the reaction mixture. The separation of the amino acids was visualized in a non-radioactive control by spraying the paper with 0.2% ninhydrin in 95% ethanol. Glutamine was found to migrate with the solvent front while glutamate remained at the origin. The number of nanomoles of ^{14}C-labeled glutamine in each sample was determined by subtracting the percentage of total radioactive counts located at the origin in the control reactions from the percentage in each sample. All values are derived from the average of four separate reactions.

To examine the effect of glutamate on the burst, wild-type AS-B was incubated on ice either alone or with 100 mM L-glutamate, or D-Glutamate for 3 hours prior to the reaction. The enzyme/glutamate mixture then was added to the reaction and product formation was determined at various time points as described above.

Hydroxylamine Partitioning of the Thioester

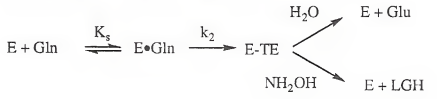
A reaction mixture containing either wt AS-B (9.3 µg), or R30A (18.5 µg), and 50 mM glutamine, 100 mM Bis-Tris and Tris-HCl (pH 8) and a variable concentration of hydroxylamine in a total volume of 300 µl was incubated at 37°C for 45 minutes. The reaction was terminated by the addition of 100 µl of 16% TCA and a 50 µl aliquot was removed to determine the amount of glutamate formed using the previously mentioned GDH assay. LGH formation was determined in a final volume of 500 µl by adding a solution containing 80% TCA, 6N HCl, and 10% $FeCl_3$ in 0.02 N HCl to the remaining reaction,

and measuring the absorbance of the hydroxamate-$FeCl_3$ complex at 540 nm. A standard curve using a stock solution of authentic LGH was used to quantitate the product formed in these reactions.

Results

Partitioning of a thioester intermediate to γ-glutamyl hydroxamate

The minimal mechanism, given in equation 1, requires the formation of a covalent intermediate. Due to the highly reactive nature of hydroxylamine towards acyl groups, this agent has been used to provide evidence for an acylenzyme intermediate and to determine whether its formation or breakdown is rate limiting in hydrolysis reactions catalyzed by serine proteases (Caplow et al., 1964; Epand et al., 1963) as well as by the amidotransferase CAD (Chaparian et al., 1991). Since hydroxylamine competes with H_2O in the deacylation step, its presence will cause an increase in the amount of total product formed (hydroxamate plus glutamate) only if this same step is rate limiting (reaction 2). In contrast, if the acylation step is very slow then the presence of hydroxylamine will not have an effect on the amount of total product produced as the slow rate of the acylation step will limit the amount of thioester available for hydrolysis and hydroxaminolysis.



(Reaction 2)

In an attempt to show that AS-B catalyzed hydrolysis occurs via a thioester intermediate and to determine which step in hydrolysis is rate limiting, the effect of various concentrations of hydroxylamine on the glutaminase reaction was examined. As shown in Figure 4.1, even very low concentrations of hydroxylamine were sufficient to partition the reaction towards the production of glutamate hydroxamate. Unexpectedly however, an increase in hydroxylamine concentration in the AS-B catalyzed glutaminase reaction caused a decrease in total product formed (figure 4.1).

One potential cause for this decrease in total product formed in the presence of hydroxylamine is a slow release of L-glutamate hydroxamate due to its tight binding to the enzyme. To test this idea, the partitioning experiment was repeated using an AS-B mutant (R30A) which exhibits poor substrate binding. A role in substrate binding has been implied for Arginine-30 in AS-B (Boehlein et al., 1994b) and the cognate arginine in GFAT (Isupov et al., 1996) in studies using site-directed mutagenesis and x-ray crystallography, respectively. Use of R30A in the partitioning experiments resulted in an increase in total product with increasing concentrations of hydroxylamine (figure 4.2).

Comparison of AS-B catalyzed hydrolysis of three substrates

The kinetic constants k_{cat}, K_m, and k_{cat}/K_m for the AS-B catalyzed hydrolysis of L-glutamine, L-glutamic acid-γ-monohydroxamate, and L-glutamic acid-γ-hydrazide are given in Table 4.1. As shown in the table, the k_{cat} values for the three substrates were similar while each displayed different k_{cat}/K_m values.

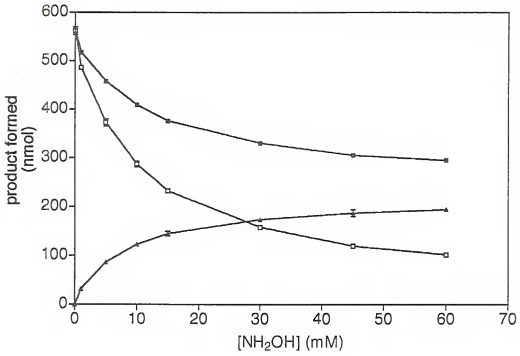


Figure 4.1: Partitioning of the thioester intermediate to L-glutamic-γ-monohydroxamate using hydroxylamine in a reaction catalyzed by wt AS-B. A mixture containing wt AS-B, 50 mM glutamine, 100 mM Bis-Tris and Tris-HCl (pH 8), and the indicated concentrations of hydroxylamine was incubated at 37°C for 45 min. The reactions were terminated by the addition of 4% TCA. An aliquot was removed to determine glutamate formed (□) by an end-point assay using glutamate dehydrogenase. LGH formation (▲) was measured by adding a $FeCl_2$ solution and measuring the absorbance at 540 nm as described in materials and methods. The nmol of LGH and glutamine formed at each concentration of hydroxylamine were added to give the total product formed (■).

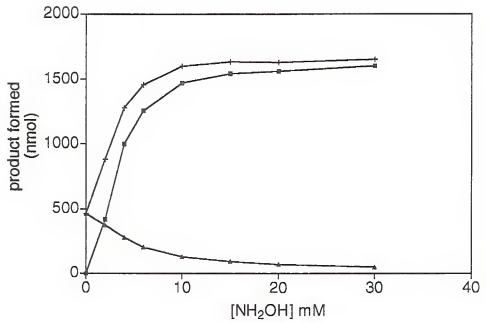


Figure 4.2: Partitioning of the thioester to glutamate hydroxamate using hydroxylamine in the glutaminase reaction catalyzed by AS-B R30A. A mixture containing wt AS-B, 80 mM glutamine, 100 mM Bis-Tris and Tris-HCl (pH 8), and the indicated concentrations of hydroxylamine was incubated at 37°C for 45 min. The reactions were terminated by the addition of 4% TCA. An aliquot was removed to determine glutamate formed (▲) by an end-point assay using glutamate dehydrogenase. LGH formation (■) was measured by adding a $FeCl_2$ solution and measuring the absorbance at 540 nm as described in materials and methods. The nmol of LGH and glutamine formed at each concentration of hydroxylamine were added to give the total product formed (+).

TABLE 4.1:
Kinetic constants for wt AS-B-catalyzed hydrolysis of L-glutamine, L-glutamic acid-γ-monohydroxamate, and L-glutamic acid-γ-hydrazide.

Substrate	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} sec^{-1})
L-glutamine	1.9 ± 0.03	1.0 ± 0.06	1900
L-glutamic-γ-monohydroxamate	2.5 ± 0.02	0.65 ± 0.02	3846
L-glutamic-γ-hydrazide	2.0 ± 0.05	2.3 ± 0.20	869

Determination of the Dissociation constants for glutamine and LGH using the covalent modifier, 6-diazo-5-oxonorleucine (DON).

The diazo ketone analogue of glutamine, DON, has been shown to inhibit the glutaminase activity of bovine AS in such a manner that the effects could be eliminated only by the addition of glutamine and not by desalting (Mehlhaff & Schuster, 1991). Furthermore, the x-ray structure of the glutamine active site labeled by DON in another class II amidotransferase, *E. coli* GPA (Kim et al., 1996), has provided evidence that this analog alkylates the cys-1 residue in a reaction which is comparable to the formation of the glutamyl-enzyme intermediate. Using the method of Meloche (1967), the affinity analogue, DON, has been used in the determination of the glutamine dissociation constant for the glutaminase reaction catalyzed by anthranilate synthetase (Goto et al., 1976), CAD glutaminase (Chaparian & Evans, 1991) and the amidotransferase PabA/PabB complex (Roux & Walsh, 1992). Likewise, the ability of DON to inactivate wild-type and the R30A mutant of AS-B was examined and the effect of glutamine or LGH on the inactivation was used to determine the dissociation constant of glutamine and LGH. Incubation of wild type AS-B with DON prior to the measurement of the glutaminase activity caused a time-dependent loss of activity over a period of 20 minutes. The semilogrithmic plot of the residual activity versus time shows that DON inactivates AS-B with pseudo-first order kinetics (figure 4.3). This plot also shows that inactivation is irreversible since the residual activity of the enzyme after it had been incubated with DON was determined using 20 mM glutamine, providing a 1400-fold dilution. The inactivation half-times, τ, were

determined from these plots and were used in a replot to determine the K_i for DON according to the following equation described by Meloche (1967):

$$\tau = \frac{1}{[I]} (TK_i) + T \qquad (1)$$

where τ is the time required for the inhibitor to cause a 50% loss of activity (τ= ln2/first order rate constant for inactivation); [I] is the inhibitor concentration; T is the time required for one-half inhibition at infinite inhibitor concentration; and K_i is the inhibition constant for the inhibitor. When measuring the residual AS-B glutaminase activity after incubating the enzyme with DON, the replot (τ vs 1/[I]) showed a straight line which extrapolated to a minimum inactivation half-time, T, of 1.22 minutes (figure 4.4). The K_i for DON determined from the slope of this replot was 34 μM.

The dissociation constant for glutamine, K_s, was determined by measuring the competitive binding of glutamine and DON. Therefore, DON inactivation was examined in the presence of 20 μM and 50 μM glutamine (Figures 4.5 and 4.6). Using the following equation (Meloche, 1967):

$$\tau = \frac{1}{[I]} TK_i \left(1 + \frac{[S]}{K_s} \right) \qquad (2)$$

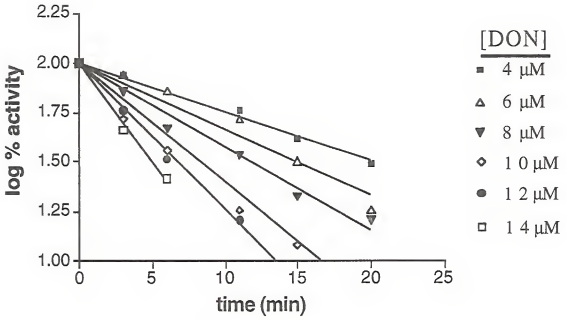


Figure 4.3: DON inactivation of AS-B in the absence of glutamine. Wild type AS-B was incubated with the concentrations of DON given in the figure. At time points ranging from 0-20 minutes, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzymes were measured as described in materials and methods.

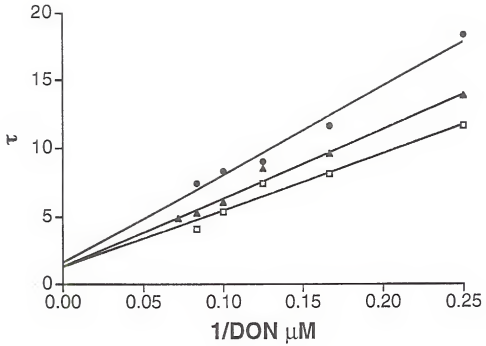


Figure 4.4: Replot of DON inactivation of AS-B in the absence (□) and presence of glutamine (▲, 20 μM; ●, 50 μM).

a replot of τ vs 1/[I] at each concentration of glutamine gave straight lines with a different slope for each glutamine concentration (Figure 4.4). The slope of the line resulting from 20 μM glutamine is 50.73 and that from 50 μM glutamine is 65.02 corresponding to K_s values of 140 μM and 200 μM respectively.

In a similar manner, DON inactivation and LGH protection against inactivation was examined for wild type AS-B, and DON inactivation and glutamine protection against inactivation was examined for the AS-B mutant R30A (Figures 4.7-4.14). Using equation (1) and the replot of τ vs 1/[DON] the minimum inactivation half-time, T, for DON inactivation of wild type catalyzed LGH hydrolysis (Figure 4.8) and R30A catalyzed glutamine hydrolysis (Figure 4.12) were 1.4 sec and 3.5 sec, respectively. The K_i for DON in these two reactions, determined from the slope of the replot was 28 μM for LGH hydrolysis and 8.7 mM for inactivation of R30A catalyzed glutamine hydrolysis. The following equation was used to determine the dissociation constant for LGH in the wild type catalyzed glutaminase reaction and for glutamine in the R30A catalyzed reaction:

$$\tau = [S]T\frac{1}{[I]}\left(\frac{K_{inact}}{K_s}\right) + \left(T + T\frac{K_{inact}}{[I]}\right) \qquad (3)$$

The K_s, derived from the slope of the line in a replot of τ vs [substrate] for LGH in the wild type catalyzed hydrolysis reaction (Figure 4.10) was 70 μM and for glutamine in the R30A catalyzed reaction (Figure 4.14) was 9.5 mM

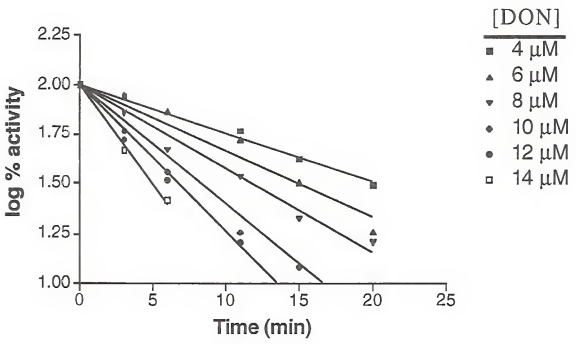


Figure 4.5: DON inactivation of AS-B in the presence of 20 μM glutamine. Wild type AS-B was incubated with the concentrations of DON given in the figure. At time points ranging from 0-20 min, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzymes were measured as described in materials and methods.

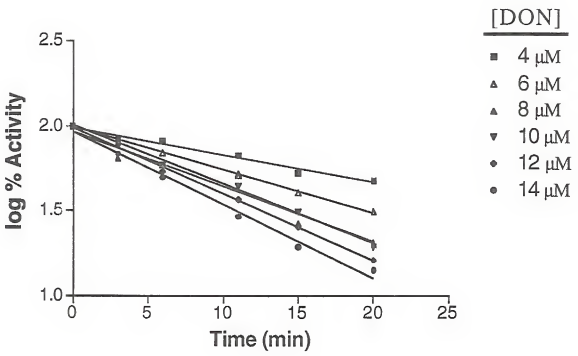


Figure 4.6: DON inactivation of AS-B in the presence of 50 μM glutamine. Wild type AS-B was incubated with the concentrations of DON given in the figure. At time points ranging from 0-20 min, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzymes were measured as described in materials and methods.

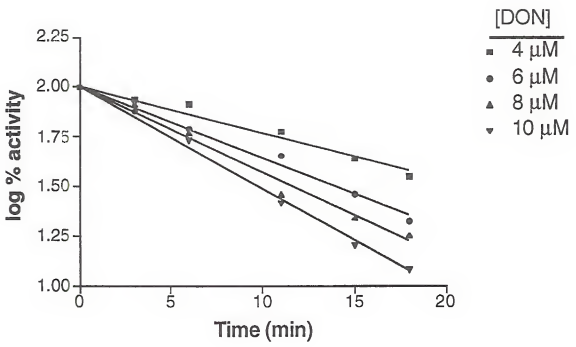


Figure 4.7: DON inactivation of wild-type catalyzed LGH hydrolysis in the absence of LGH. Wild type AS-B was incubated with the concentrations of DON indicated in the figure. At time points ranging from 0-18 min an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzymes were measured as described in materials and methods.

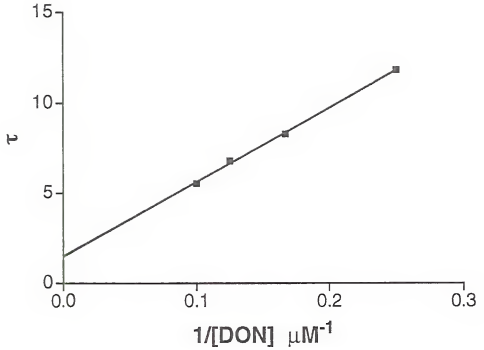


Figure 4.8: Replot of DON inactivation of AS-B catalyzed LGH hydrolysis. The K_i for DON, determined from the slope of this plot and equation (1), was 28 μM.

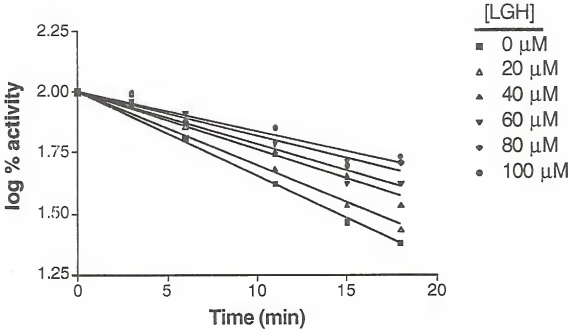


Figure 4.9: The effect of various concentrations of LGH on DON inactivation of AS-B catalyzed LGH hydrolysis. Wild-type AS-B was incubated with 6 μM DON in the presence of LGH (concentrations varied as indicated in the figure). At time points ranging from 0-18 min, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM LGH. The LGH hydrolysis activity of the DON modified enzymes were measured as described in materials and methods.

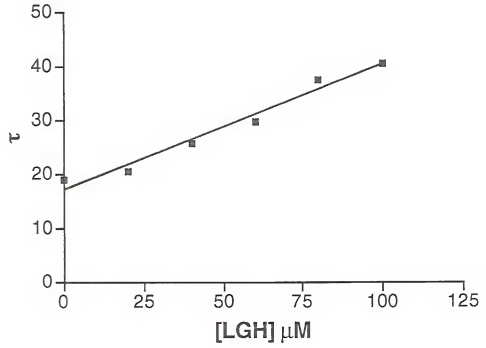


Figure 4.10: LGH protection against DON inactivation of AS-B catalyzed LGH hydrolysis. The K_s , determined from the slope of the line and equation (3), was 70 μM.

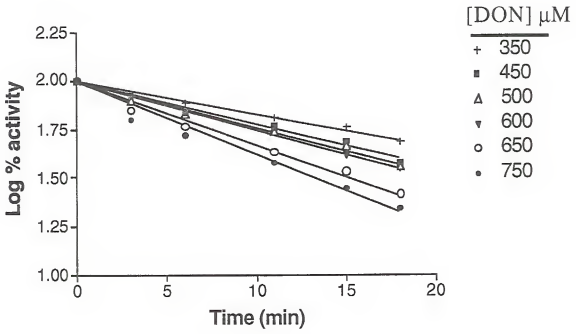


Figure 4.11: DON inactivation of the R30A mutant of AS-B in the absence of glutamine. R30A was incubated with the concentrations of DON given in the figure. At time points ranging from 0-18 min, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzyme were measured as described in materials and methods.

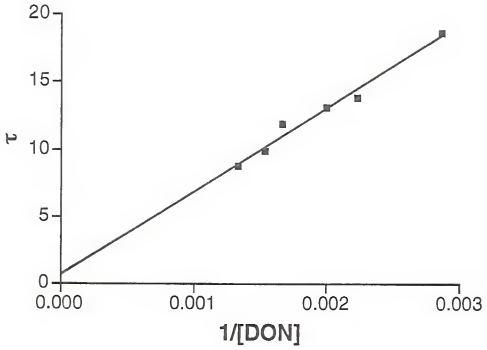


Figure 4.12: Replot of DON inactivation of R30A in the absence of glutamine. The K_i for DON, determined from the slope of the line and equation (1), was 8.7 mM.

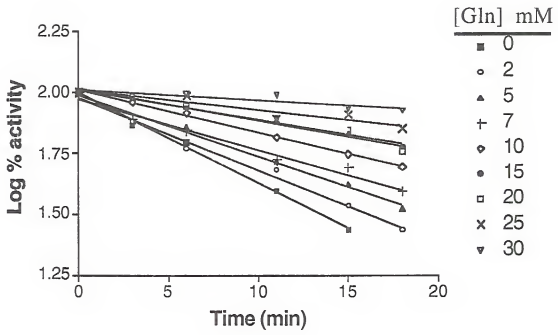


Figure 4.13: Glutamine protection from DON inactivation of the R30A catalyzed glutaminase reaction. R30A was incubated with 0.65 mM DON and various concentrations of glutamine (given in the figure). At time points ranging from 0-18 min, an aliquot was removed from the mixture and tested for residual activity in a reaction containing 12 mM glutamine. The glutaminase activities of the DON modified enzyme were measured as described in materials and methods.

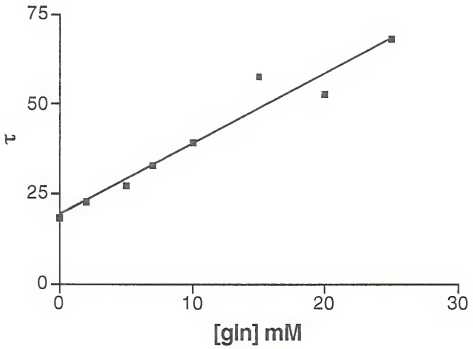


Figure 4.14: Replot of glutamine protection of DON inactivation of R30A catalyzed glutaminase. The K_s, determined from the slope of the line and equation (3), was 9.5 mM.

A step after the formation of the thioester intermediate is rate-determining in glutamine hydrolysis catalyzed by AS-B

In order to determine which step is rate limiting in the minimal mechanism, the production of glutamate was studied as a function of time. A burst of product formation during the first turnover of the enzyme is an indication that the slow step follows the appearance of the thioester intermediate.

The time-course of glutamate production was examined at 5°C, a temperature at which the reaction was slow enough to mix by hand. A graph of nmol of glutamate formed versus reaction time (figure 4.15) shows that the rate limiting step occurs after the formation of the thioester intermediate as extrapolation of the line to time zero intercepts the y-axis at a point above zero. This point of intersection should represent the concentration of the dominant intermediate, possibly the thioester, and therefore a time course using a higher concentration of enzyme should intercept the y-axis at a higher value. In fact, a higher y-axis intercept is observed with double the concentration of enzyme (Figure 4.15). The burst of product formed in the first turnover of an enzymatic reaction occurs because the steps leading to product formation are fast relative to those which follow. In the minimal mechanism proposed for AS-B, the first appearance of glutamate occurs upon the formation of the thioester intermediate. Therefore, it should be possible to eliminate the appearance of the burst by forcing the reaction to begin at an enzyme form which would require passing through the rate determining step prior to the first appearance of glutamate. As shown in Figure 4.16, the burst in glutamate formation was abolished

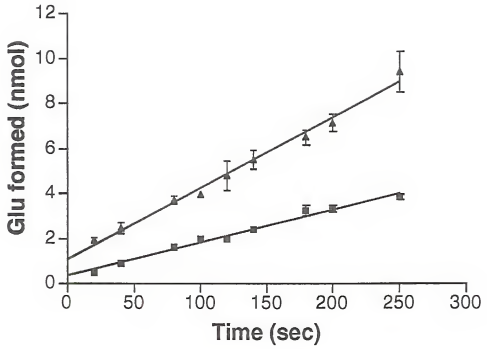


Figure 4.15: Glutamate formation in AS-B catalyzed glutamine hydrolysis as a function of time. The progress curve shows the nmol of glutamate formed versus time using 2 mM [^{14}C]-glutamine (SA=9nCi/µl), and either 0.37 nmol (■) or 0.74 nmol (▲) of wt AS-B in 100 mM Bis-Tris and Tris-HCl (pH 8). After terminating the reaction with acetic acid, the glutamine and glutamate were separated by anion exchange chromatography as described in the materials and methods and the radioactivity associated with each amino acid was counted. All values are derived from the average of four separate determinations.

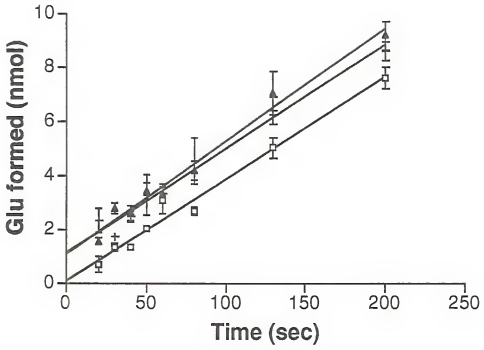


Figure 4.16: Effect of the presence of L-glutamate on the burst in product formation in AS-B catalyzed glutamine hydrolysis. The figure shows the nmol of glutamate formed versus time in reactions where wild-type AS-B was incubated alone (+) or with 100 mM L-glutamate (□) or D-glutamate (▲) prior to addition into the reaction.

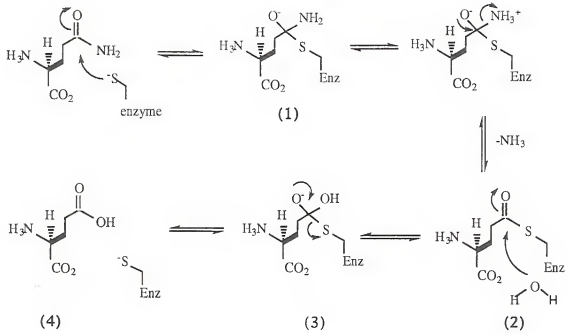


Figure 4.17: Mechanism of glutamine hydrolysis catalyzed by the class II glutamine amidotransferases as proposed by Mei and Zalkin (1989).

in a time course experiment in which the enzyme was incubated with L-glutamate prior to its addition to the reaction. The observation that D-glutamate has no effect on the burst suggests that salt effects are not a cause of the disappearance of burst in the presence of L-glutamate.

Discussion

This report describes the characterization of the AS-B catalyzed glutaminase reaction by a comparison of its kinetic behavior to that of papain and CAD. The mechanism previously proposed for glutamine hydrolysis catalyzed by the class II amidotransferases (Mei & Zalkin, 1989) (figure 4.17), in analogy to that of papain, involves a nucleophilic attack of the N-terminal cysteine on the side chain of glutamine to form a tetrahedral intermediate (**1**). Subsequent protonation of the leaving group allows the release of ammonia and the formation of a thioester intermediate (**2**). An attack of a water molecule on the thioester then causes the formation of a second tetrahedral intermediate (**3**) and finally the production of free enzyme and glutamate (**4**). Using the minimal model in equation 1, it has been found that for papain the formation of the thioester (k_2) is rate limiting when amide substrates are used. This observation is probably due to the greater leaving group ability of the thiol group relative to the amine or the hydroxyl group of the first and second tetrahedral intermediate, respectively (O'Leary et al., 1974; Wells et al., 1963). This situation causes the reaction to favor return from the first tetrahedral intermediate to free enzyme and substrate whereas it favors forward progression from the second tetrahedral intermediate to free enzyme and product.

In contrast to the above observations for papain, evidence is presented in this study for a rate limiting thioester breakdown for AS-B catalyzed glutamine hydrolysis. The similarity in the k_{cat} values for three substrates with different leaving groups suggests that a step following the release of the leaving group and thus after the appearance of a common intermediate is rate limiting (Table 4.1). Moreover, a burst in glutamate production with time indicates that an intermediate containing a glutamate moiety is formed quickly followed by a slow breakdown to product and free enzyme (Figure 4.15). The presence of the burst in the production of glutamate was verified by the ability to eliminate it when the enzyme was forced into an enzyme-product form (Figure 4.16). It is possible that glutamate could bind the enzyme and form a thioester intermediate, therefore the disappearance of the burst in the presence of glutamate does not reveal which step after thioester formation is rate limiting. On the other hand, under the acidic conditions of the reaction termination (see materials and methods), it is likely that the acylenzyme intermediate remained intact and precipitated in the acidic solution. Therefore, the radioactive glutamate counted after paper chromatography represented only the glutamate which was not covalently attached to the enzyme. If this is true, then the burst suggests that a step occurring after deacylation is rate limiting. Further work to address this issue will be presented in subsequent chapters.

While the experiments described above suggest a rate determining step following the formation of a thioester intermediate, the results from hydroxylamine partitioning studies were more

complicated. Indeed, additional evidence for the presence of a thioester intermediate along the reaction pathway of AS-B catalyzed glutamine hydrolysis is given in the formation of LGH upon addition of hydroxylamine to the glutaminase reaction. However, an increase in the hydroxylamine concentration in the reaction was expected to either increase the total product formed, if thioester breakdown (k_3) were rate limiting, or to have no effect on the amount of product formed, if thioester formation (k_2) were rate limiting. For example, hydroxylamine partitioning experiments have been used to show that deacylation is rate limiting in glutamine hydrolysis catalyzed by a class I amidotransferase, CAD (Chaparian & Evans., 1991). In contrast, an increase in hydroxylamine concentration in the AS-B catalyzed glutaminase reaction resulted in an unexpected decrease in total product formed (Figure 4.1). This observed decrease in total product may have been caused by hydroxylamine inhibition of the enzyme. However, the K_m for hydroxylamine in an LGH synthesis reaction (see chapter 3) has been shown to be several times higher than the concentration used in these experiments. Alternatively, LGH release from the enzyme may be very slow thus hindering the regeneration of free enzyme for further hydrolysis. Consistent with this hypothesis, an R30A mutant of AS-B with impaired substrate binding (shown in its K_s, Figure 4.2) exhibited an increase in total product formed with increasing concentrations of hydroxylamine indicating a rate limiting deacylation step (Figure 4.2).

The findings described in this report represent mechanistic differences between substrate hydrolysis catalyzed by the class II amidotransferase, AS-B, and hydrolysis catalyzed by the thiol

protease, papain, and the class I amidotransferase, CAD. While both AS-B and CAD exhibit a rate determining step occurring after the formation of the acyl enzyme, a feature unlike the slow acylation step in amide hydrolysis catalyzed by papain, AS-B may have an additional step which contributes to the rate of the reaction and is unlike the sole rate determining deacylation which characterizes CAD catalysis (Chaparian & Evans, 1991). Additional effort will be required to understand the details of the mechanisms of these reactions which cause the differences seen in the rate determining steps. One step towards accomplishing this goal is a knowledge of the rates of the individual steps in the reactions. In this regard, the K_s for glutamine determined in this work not only reveals that the glutaminase reaction occurs under rapid equilibrium conditions ($k_{-1}>>k_2$) but also contributes a rate constant to the overall model of the reaction which will be presented in Chapter 7. In addition, Chapters 5 and 6 represent the first attempt at the examination of an individual step in the reaction.

CHAPTER 5
EVIDENCE OF A THIOESTER INTERMEDIATE FORMED DURING GLUTAMINE HYDROLYSIS CATALYZED BY ASPARAGINE SYNTHETASE B

Introduction

Numerous clinical and basic studies have correlated the importance of asparagine biosynthesis with the ability of some leukemia cells to escape the toxic effects of treatment with L-asparaginase (Chakrabarti & Schuster, 1997). As a result of this relationship, a great deal of effort has been directed towards understanding the chemical and kinetic mechanism of asparagine synthetase, the enzyme mediating asparagine formation (Richards & Schuster, 1997). It has been the goal of several research groups to obtain specific, and potent, inhibitors of AS that might prevent asparagine synthesis in tumor cells, and therefore act as either adjuncts to L-asparaginase therapy or replacements for this drug (Cooney et al., 1976; Cooney et al., 1980). Such efforts have been hampered, however, by an incomplete understanding of the chemical mechanism by which nitrogen is removed from the side chain amide of glutamine and transferred to ß-aspartyl-AMP to form asparagine (Richards & Schuster, 1997).

Escherichia coli asparagine synthetase B (AS-B) (Boehlein et al., 1994a; Scofield et al., 1990) is a member of the class II (formerly *purF*) amidotransferase superfamily (Smith, 1995; Zalkin, 1993), that

includes glutamine 5'-phosphoribosylpyrophosphate amido-transferase (GPA) (Tso et al., 1982), glutamine fructose-6-phosphate amidotransferase (GFAT) (Badet-Denisot et al., 1993), and glutamate synthase (Vanoni et al., 1991). These enzymes are characterized by a highly conserved N-terminal cysteine residue in their mature, active form. AS-B catalyzes the ATP dependent synthesis of asparagine utilizing either ammonia (Reaction 1) or glutamine (Reaction 2) as a nitrogen source. When aspartate is absent, AS-B catalyzes the hydrolysis of glutamine to glutamate and ammonia (Reaction 3), in a reaction that is stimulated by ATP (Boehlein et al., 1994b; Boehlein et al., 1996).

Reaction 1: L-Asp + NH_3 + ATP ---> L-Asn + AMP + PPi

Reaction 2: L-Asp + L-Gln + ATP --> L-Asn + L-Glu + AMP + PPi

Reaction 3: L-Gln --> L-Glu + NH_3

AS-B has been employed in an extensive series of studies aimed at delineating (i) the molecular mechanism of asparagine synthesis, and (ii) the functional roles of conserved residues in glutamine binding and activation (Boehlein et al., 1994a; Boehlein et al., 1994b; Boehlein et al., 1996; Richards & Schuster, 1997). The high level of glutaminase activity exhibited by AS-B has also been exploited (Boehlein et al., 1997b) to show a mechanistic relationship between Class II amidotransferases and thiol proteases, such as papain (Storer & Menard, 1994). On the other hand, and despite substantial effort (Badet, B., personal communication), the thioester intermediate that must therefore be formed during glutamine breakdown has not yet

been characterized for any member of the Class II amidotransferase family. We now report experimental conditions for the isolation of a γ-glutamyl AS-B adduct, together with evidence supporting the hypothesis that this covalent derivative is the putative thioester intermediate formed during AS-B catalyzed glutamine hydrolysis. The characterization of this intermediate is likely to allow further insight into the details of the kinetic and chemical mechanisms of nitrogen transfer in AS-B, and other Class II amidotransferases.

Materials and Methods

Enzymes and reagents.

Recombinant wild-type AS-B and the C1A AS-B mutant, in which Cys-1 is substituted by alanine, were constructed, expressed and purified using published procedures (Boehlein et al., 1994a). Protein concentration was determined using an assay kit supplied by BioRad and immunoglobulin G as a standard. Recent examination of the protein concentration by amino acid analysis of AS-B, performed by the Protein Core Facility of ICBR at the University of Florida, gave a value for protein concentration which was 2.7-fold lower than that obtained with the previous method. Therefore, the enzyme concentration has been corrected by a factor of 2.7. [U-^{14}C]-L-Glutamine (277 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). 6-Diazo-5-oxo-L-norleucine (DON) and Sephadex G-50 Fine were obtained from Sigma Chemical Company.

Isolation of a Covalent Adduct between AS-B and [^{14}C]-glutamine

Wild-type AS-B (0.74 nmol) or the C1A AS-B mutant (0.74 nmol) was incubated with 1 mM [U-^{14}C]-L-glutamine (SA 22,000

dpm/nmol) in 100 mM Tris-HCl (pH 8.0) at room temperature (total reaction volume 100 µl). After 30 s, the reaction was quenched in 1 ml of 8% TCA on a 2.5 cm nitrocellulose filter (0.45 mm porosity) and 100 µl BSA (10 mg/ml) was added. After 2 minutes in the quench solution the samples were filtered under vacuum on a 2.5 cm nitrocellulose filter (0.45 µm porosity), and the filter was washed with 50 ml of 1 N HCl. The filter was transferred to 5 ml of scintillation fluid (ScintiVers II*) and the ^{14}C activity was measured on a Beckman model LS6000IC scintillation counter. This procedure was repeated in the absence of enzyme as a control for non-specific binding of L-glutamine to the filter.

Gel Filtration of the Intermediate formed between AS-B and [^{14}C]-Glutamine

(A) 1 mM [U-^{14}C]-L-glutamine (SA 22 000 dpm/nmol) was incubated with either wt AS-B (0.74 nmol) or the C1A AS-B mutant (0.74 nmol) in 100 mM Tris-HCl (pH 8.0) at room temperature (total reaction volume 100 µl). After 30 s, reactions were terminated using 22 µl of 0.1 M NaOAC (pH 4.0) containing 5% SDS, and the protein was separated from free glutamine by gel filtration using Sephadex G-50 Fine spin column equilibrated with 1% SDS in 0.1 M NaOAc (pH 4.0) (Penefsky, 1979). The amount of radiolabeled enzyme was then measured by liquid scintillation counting. (B) In a separate gel filtration experiment, wt AS-B (2 nmol) was incubated with 2 mM [U-^{14}C]-L-glutamine (SA 11,000 dpm/nmol) in 100 mM Tris-HCl (pH 8.0) for 30 s at room temperature (total volume 100 µl). After termination by dilution with 100 µl of 8M guanidinium-HCl in 0.1M NaOAc (pH 4.0), the protein was isolated by gel filtration on a

Sephadex G-50 Fine spin column (Penefsky, 1979) equilibrated in the quench solution. The amount of radiolabeled enzyme was measured by liquid scintillation counting.

Lability of the intermediate formed between AS-B and [^{14}C]-Glutamine at high pH

The gel filtration procedure was used to determine the stability of the intermediate at low and high pH. Wild-type AS-B (4.8 nmol) was incubated 30 s at room temperature with 1.5 mM [^{14}C]-Glutamine (25,633 dpm/nmol) in a 100 µl reaction containing 100 mM Bis-Tris and Tris-HCl (pH 8). The reactions were terminated by the addition of 22 µl 0.5 M sodium acetate (pH 4) in 5% SDS. The protein was separated from free glutamine by the method of Penefsky (1979) using a Sephadex G-50 fine spin column equilibrated in either: 1) 0.1 M sodium phosphate (pH 12) in 1% SDS or 2) 0.1 sodium phosphate (pH 2) in 1% SDS. The amount of radiolabeled enzyme in 70 µl of the solution which passed through the column was measured by liquid scintillation counting, and the protein concentration was measured using an assay kit provided by BioRad. Values for the protein concentration and the percent of enzyme labeled at each pH were derived from seven separate experiments.

Results

The partitioning studies presented in chapter 4 provided evidence for a thioester formed on the reaction pathway of glutamine hydrolysis catalyzed by wt AS-B. If the breakdown of the thioester intermediate is slower than its formation, as indicated in

the previous chapter, then accumulation of this intermediate may allow an opportunity for its isolation.

Two different methods were used to isolate a covalent adduct formed between wt AS-B and glutamine. In the initial approach, labeled protein was obtained by incubating wt AS-B with [^{14}C]-L-glutamine, denaturing the protein with SDS in acid solution, and separating the enzyme from free glutamine by gel filtration (Table 1). The radioactivity associated with the enzyme most likely was not due to non-specific glutamine binding as wt AS-B that had been covalently modified by 6-diazo-5-oxonorleucine (DON), prior to incubation with [^{14}C]-L-glutamine under these conditions, did not give the radiolabeled covalent adduct (Table 5.1). Furthermore, radiolabeled protein was not formed when [^{14}C]-L-glutamine was incubated with the C1A AS-B mutant in place of the wild-type enzyme, despite the observation that this AS-B mutant binds L-glutamine with high affinity (Boehlein et al., 1994a).

While gel filtration could be used successfully to isolate the putative γ-glutamyl AS-B adduct, quantitative measurements of its kinetic properties were hampered by a reduction in the amount of radiolabeled enzyme as a function of the time during which samples were maintained in the quench solution (Table 5.2). After approximately fifteen minutes, the observed reduction in radiolabeled protein stabilized at 59% of the value observed at short incubation times. Efforts to address this problem by employing 8 M guanidine-HCl as a denaturant, instead of SDS, did not affect the rate of loss of radiolabeled enzyme.

In contrast to the slow disappearance of the γ-glutamyl-enzyme adduct in the gel filtration approach, the radiolabeled protein was stable when isolated by filter binding (Lusty, 1992), and could be used in quantitative measurements. In these experiments, enzyme was combined with [^{14}C]-L-glutamine, denatured in TCA, and the precipitate filtered and washed with HCl. The radioactivity remaining on the filter was measured using liquid scintillation counting. Approximately 35% of the enzyme was radiolabeled in reaction mixtures containing wild-type AS-B (Table 5.3). Examination of the effect of glutamine concentration on the steady-state level of the glutamyl-enzyme complex revealed a saturation curve which gave a maximum molar ratio of intermediate to enzyme of 0.14 and a K_M of 0.14 mM for glutamine (Figure 5.1). In control studies, radioactivity was not associated with the filter when incubation solutions lacked the enzyme, or contained either the C1A AS-B mutant or wild-type AS-B that had been covalently modified with DON (Table 5.3).

The stability of thioesters decreases with increasing pH, therefore if the isolated intermediate is a thioester the radioactivity associated with the enzyme should diminish under alkaline conditions. Initially, an attempt was made to determine the stability of the intermediate at various conditions of pH using the filter binding assay. Unfortunately, the enzyme redissolved under the alkaline conditions of the wash and passed through the filter. Thus, the observed disappearance of radioactivity associated with the membrane was due to the decrease in enzyme on the filter rather than instability of a thioester intermediate. As an alternative

approach, the adduct was formed, the reaction was terminated, and the resulting solution was added to a gel filtration column either equilibrated at pH 2 or pH 12. As shown in Table 5.4, 35% of the enzyme was labeled after gel filtration at pH 2 whereas only 10% of the enzyme was labeled after gel filtration at pH 12. While a decrease in radiolabeled enzyme isolated by gel filtration was mentioned above, this problem was alleviated by maintaining a constant time interval of 20 s between terminating the reaction and addition of the solution to the column. As shown through the statistics at each pH, variability in the amount of radiolabeled enzyme due to slow termination does not seem to be a factor.

Discussion

Glutamine amidotransferases can be divided into two families based on multiple sequence alignment of the glutamine amide transfer (GAT) domains (Zalkin, 1993). Class I (formerly *TrpG*) enzymes, such as GMP synthetase (Tesmer et al., 1996) and carbamoyl phosphate synthetase (CPS) (Thoden et al., 1997) are characterized by conserved Cys, His and Asp active site residues that are thought to function as a catalytic triad in a manner analogous to thiol proteases (Brocklehurst et al., 1987). Thus, in analogy to the papain mechanism (Brocklehurst et al., 1987), glutamine hydrolysis proceeds by initial attack of the Cys thiolate on the side chain amide to yield a tetrahedral intermediate (Scheme 1). Breakdown of this intermediate after N-protonation, by the conserved active site histidine to transform the nitrogen into an efficient leaving group, then yields a thioester. Subsequent hydrolysis of this intermediate

would then regenerate the enzyme and produce glutamate. Direct support for this mechanistic hypothesis for Class I amidotransferases has been provided by isolation of the thioester in studies on CAD (Chaparian & Evans, 1991), *Escherichia coli* PabA (Roux & Walsh, 1992) and CPS (Lusty, 1992; Wellner et al., 1973). This accomplishment has contributed significantly to understanding the mechanism of glutamine hydrolysis in these enzymes by allowing use of a minimal model to examine the rate determining step of the reaction, as well as the effects of the other substrates on this step.

Class II amidotransferases, on the other hand, possess a conserved N-terminal cysteine that has been demonstrated to be essential for all glutamine-dependent activities of these enzymes (Badet et al., 1987; Boehlein et al., 1994a; Mei & Zalkin, 1989; Van Heeke & Schuster, 1989). Although early experiments suggested the presence of a catalytically important histidine in GPA (Mei & Zalkin, 1989) site directed mutagenesis of histidines within the GAT domain of AS-B has almost no effect on the kinetic properties of the resulting mutant enzymes (Boehlein et al., 1994a) and recent X-ray crystal structures of *Bacillus subtilis* GPA (Smith et al., 1994), *Escherichia coli* GPA (Kim et al., 1996) and an N-terminal fragment of *Escherichia coli* GFAT (Isupov et al., 1996) do not reveal a histidine in the appropriate position to participate in acid-base catalysis. Class II amidotransferases have therefore been proposed to be members of the N-terminal nucleophile (Ntn) hydrolase superfamily (Brannigan et al., 1995), that includes the 20S proteasome (Seemuller et al., 1996) and penicillin acylase (Duggleby et al., 1995). Ntn hydrolases are characterized by an N-terminal catalytic nucleophile (hydroxyl or

thiol) that is thought to be activated by transfer of its proton to the free N-terminal amino group (Brannigan et al., 1995). Thus, in the case of AS-B and other Class II amidotransferases, the α-amino group of Cys-1 would serve as a general acid-base catalyst in place of histidine. No unambiguous kinetic evidence, however, has yet been reported that supports such an assumption. The observation of a significantly smaller amide ^{15}N (V_{max}/K_m) kinetic isotope effect for AS-B catalyzed glutamine hydrolysis compared to that observed for papain catalyzed amide hydrolysis, however, may reflect this difference in the nature of protonation of the leaving group in the tetrahedral intermediate (Stoker et al., 1996). Whereas in papain, His-159 functions as a general acid catalyst (Brocklehurst, et al., 1987) it has been suggested that, at least for AS-B, N-protonation occurs by specific acid catalysis (Stoker et al., 1996). On the other hand, Class II amidotransferases do exhibit many characteristics of thiol proteases, including an oxyanion hole (Boehlein et al., 1994a; Boehlein et al., 1997; Isupov et al., 1996; Kim et al., 1996).

In light of these structural and kinetic differences, it is important to validate the assumption of a thioester intermediate in AS-B catalyzed glutamine hydrolysis. Toward this goal, the demonstration of the formation of LGH upon addition of hydroxylamine to the glutaminase reaction presented in this report supports the notion that a thioester is formed on the main pathway of the hydrolysis reaction catalyzed by AS-B. Since the amide of glutamine is resonance stabilized, hydroxylamine attack requires its conversion to a more activated carbonyl derivative. Several intermediates other than a thioester could serve as this activated

intermediate. For example, a carboxyl group on the enzyme could attack the side chain of glutamine to form an anhydride intermediate. In this case, hydroxylamine could attack on either the glutamyl or enzyme side of the central oxygen of this intermediate. Therefore, each attack of the hydroxylamine on the enzyme side would result in a covalently labeled enzyme which would be unavailable for further turnover thus resulting in inhibition of the reaction with time. Since a time dependent inhibition of the reaction in the presence of hydroxylamine was not observed and since an essential glutamate or aspartate residue which could participate in forming such an intermediate has never been found in AS-B, a mechanism involving an anhydride intermediate is unlikely. Alternatively, LGH could be formed via a pyroglutamate although this would not be efficient in ammonia transfer. The possibility of an oxygen ester intermediate rather than a thioester is also unlikely since hydroxylamine causes partitioning even at pH 6 which is consistent with a thioester and not the less reactive oxygen ester which only reacts with hydroxylamine at alkaline pH. The demonstration of a radiolabeled intermediate upon incubation of wt AS-B with [^{14}C]-glutamine provides further evidence for an acylenzyme intermediate. Several observations support that this isolated adduct is in fact the thioester. First, a radiolabeled enzyme was not formed when the wild-type enzyme was labeled with DON prior to the reaction (Tables 5.1 & 5.3). DON has been shown to react at the active site of mammalian asparagine synthetases (Mehlhaff & Schuster, 1991; Larsen & Schuster, 1992) and causes irreversible enzyme inactivation by modification of the thiol of Cys-1, as

observed in the crystal structure of DON-inactivated *Escherichia* coli GPA (Kim et al., 1996). Second, radiolabelled enzyme was not found when the C1A AS-B was incubated with [^{14}C]-L-glutamine in place of wild-type enzyme (Tables 5.1 & 5.3). This AS-B mutant lacks both glutamine-dependent synthetase and glutaminase activity but retains ammonia dependent activity and the ability to bind glutamine tightly (Boehlein et al., 1994a). Since previous studies employing asparagine synthetases, as well as other Class II amidotransferases, have demonstrated the involvement of the Cys-1 side chain in formation of the thioester linkage, the lack of a γ-glutamyl C1A complex strongly suggests that covalent modification occurs at the GAT-domain active site requiring the Cys-1 thiolate. The failure of the C1A AS-B mutant to form a radiolabeled complex is further evidence that non-specific glutamine binding cannot account for the radioactivity associated with the wild-type enzyme. Third, the amount of intermediate saturates with increasing glutamine concentration, the half saturation point being consistent with steady state parameters observed for the AS-B glutaminase reaction (Figure 5.1). And finally, the intermediate was isolated only under acidic conditions and was not stable at higher pH which is consistent with the alkaline lability of thioesters.

Although the covalent γ-glutamyl AS-B adduct appeared unstable under the denaturation conditions used in the gel filtration assay, possibly reflecting residual activity of slowly denaturing enzyme in the solutions containing SDS or guanidinium HCl, approximately 34% of the enzyme was radiolabeled based on data from filter-binding. The percentage of enzyme in the thioester form,

which was similar to that reported for PabA (Roux & Walsh, 1992) indicates that this intermediate is kinetically important.

The isolation of the intermediate described here is an essential step in obtaining an accurate model of glutamine hydrolysis catalyzed by Class II amidotransferases. Furthermore, the ability to detect and quantitate this intermediate has important implications in determination of the mechanism of glutamine-dependent nitrogen transfer for AS-B and other enzymes in this family. For asparagine synthetases, current experimental evidence suggests that nitrogen transfer can proceed via direct attack of the tetrahedral intermediate (Scheme 1) on ß-aspartyl-AMP, or by release of enzyme-bound, unprotonated ammonia from glutamine (Richards & Schuster, 1997). While these mechanisms of glutamine amide transfer are substantially different, the formation of a thioacylenzyme intermediate during asparagine synthesis is a common requirement. Methods for isolation of the thioester therefore have potential application in experiments aimed at resolving which of these two transfer mechanisms is operative in AS-B. For example, if the thioester intermediate is formed prior to the breakdown of ß-aspartyl -AMP, then nitrogen transfer mediated by enzyme-bound ammonia is mechanistically most likely. On the other hand, if no thioester is formed until after ß-aspartyl-AMP breakdown, then it is most probable that direct amide transfer takes place via covalent intermediates.

TABLE 5.1

Isolation of a covalent glutamyl-enzyme intermediate by gel filtration.

Enzyme	dpm
wild-type (5 nmol)	5016 ± 100
wild-type (4 nmol) + 1 mM DON	152 ± 52
C1A (4 nmol)	65 ± 2

The intermediate was formed by incubating the indicated concentration of enzyme with 1 mM [^{14}C] glutamine (S.A. 22,000 dpm/nmol) as described in the materials and methods. The reactions were terminated with 22 µl of 5% SDS in 0.1 M sodium acetate (pH 4.0) and the enzyme was separated from free glutamine using a Sephadex G-50-80 spin column equilibrated in 1% SDS in 0.1 M sodium acetate (pH 4.0) according to the method of Penefsky (1979). In the second entry, wt AS-B (1.5nmol) was incubated with 1mM DON for 30 min prior to dilution into the reaction mixture containing [^{14}C] glutamine. The amount of radiolabeled enzyme in the column effluent was then measured by liquid scintillation.

TABLE 5.2

Time-dependence of γ-glutamyl AS-B adduct degradation in SDS solution.

Time (min)	dpm
< 1	4802 ± 77
1	4165 ± 3
5	3593 ± 460
15	2828 ± 220
29	3036 ± 72

The intermediate was formed using 4 nmol of wild-type AS-B and 1 mM [^{14}C] glutamine (S.A. 22,000 dpm/nmol). The reactions were terminated as described in Table I and then incubated for the amount of time indicated before they were added to the sephadex column.

TABLE 5.3

Trapping the thioester by filter binding

Sample	dpm
- enzyme	384 ± 187
C1A	290 ± 115
wt + DON	185 ± 55
wt	6504 ± 183

The intermediate was formed as described in materials and methods using 2 nmol of enzyme and 1 mM glutamine (S.A. 22,000 dpm/nmol). To examine the effect of DON on intermediate formation, 1mM DON was incubated with wild-type AS-B for 30 minutes. The enzyme covalently labeled with DON was then diluted into the reaction mixture containing [^{14}C] glutamine.

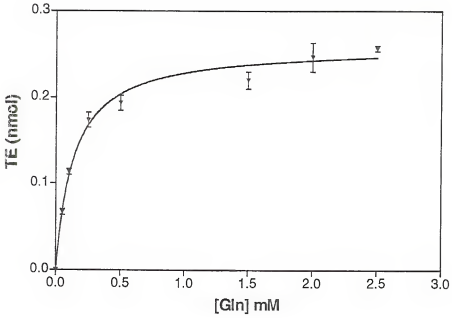


Figure 5.1 The effect of glutamine on the steady-state concentration of the thioester. Various concentrations of [^{14}C] glutamine were combined with wild-type AS-B (0.74 nmol) and the covalent adduct was isolated using a filter binding method as described in Materials and Methods.

Table 5.4

Base lability of the covalent adduct isolated by gel filtration.

Conditions	Protein Concentration (mg/ml)	Enzyme Labeled (%)
pH 2	1.18 ± 0.06	37 ± 2
pH 12	1.6 ± 0.2	10 ± 2

In all reactions, wt AS-B (4.8 nmol) was incubated 30 s at room temperature with 1.5 mM [^{14}C]-glutamine (25,633 dpm/nmol) in a reaction containing 100 mM Bis-Tris and Tris-HCl (pH 8). The reactions were terminated by the addition of 22 µl 0.5 M sodium acetate (pH 4) in 5% SDS. To determine the stability of the intermediate at pH 2 and 12, the enzyme was separated from free glutamine by the method of Penefsky (1979) using a Sephadex G-50 fine spin column equilibrated in 0.1 M sodium phosphate (either pH 2 or pH 12) in 1% SDS. The amount of radiolabeled enzyme in 70 µl of the solution which passed through the column was then measured by scintillation counting, and the protein concentration was measured using an assay kit supplied by BioRad.

CHAPTER 6
CHARACTERIZATION OF A THIOESTER INTERMEDIATE

Introduction

The ability to isolate an intermediate on the reaction pathway of glutamine hydrolysis catalyzed by AS-B (Chapter 5) will provide an opportunity to gain a more detailed description of the reaction by examining the rate of the individual step of deacylation. Previous chapters have suggested that the rate determining step of the reaction occurs after the formation of the covalent intermediate, but that the deacylation step may not be the slow step. Therefore, knowledge of the rate of deacylation will allow a comparison with the overall rate of the reaction. If indeed, deacylation is not rate limiting then the rate of this step should be significantly faster than that of the overall reaction.

The ability to measure the rate of deacylation also provides a tool with which to determine the significance of conserved residues in an individual step of the reaction. The importance of Cys-1 in making the thioacyl bond has already been implied in chapter 5 by the inability to capture a glutamyl-enzyme intermediate when this cysteine residue is replaced with an alanine (Chapter 5). Two other residues, conserved among the class II amidotransferases, which may participate in either the acylation or deacylation process are Asn-74 and Arg-30. Evidence is mounting that Asn-74 plays a role,

very similar to Gln-19 of papain, as part of an oxyanion hole which stabilizes the tetrahedral intermediates thought to be formed during the acylation and deacylation steps in the hydrolysis reaction (Boehlein et al., 1994b; 1996; 1997). Therefore, such an involvement should be reflected in a difference in either the steady-state concentration of the thioester or the rate of deacylation catalyzed by the AS-B mutant, N74A, relative to those values measured for the wt enzyme.

Several roles have been suggested for Arginine-30 such as an involvement in the stabilization of the glutamine binding site, the communication between the GAT and synthetase domains, and the release of nitrogen from glutamine (Boehlein et al., 1994b). These ideas were supported by structural information obtained for GPA (Kim et al., 1996) and GFAT (Isupov et al., 1996) which revealed a network of hydrogen bonding between the cognate arginine residue in these enzymes and other residues involved in catalysis in the glutamine site including Cys-1 and Asn-101(Asn-74 in AS-B). While chapter 4 presented evidence which supports the active participation of Arg-30 in glutamine binding, additional exploration into its interactions and functions will be necessary to obtain a more precise and comprehensive picture of its purpose. For example, partitioning studies (Chapter 4) suggested that the deacylation step is rate limiting in glutamine hydrolysis catalyzed by the R30A mutant of AS-B. This conclusion can now be tested by comparison of the rate of deacylation (determined by direct measurement) with that of the overall reaction.

In the previous chapter, the steady-state concentration of the putative thioester intermediate, formed during glutamine hydrolysis catalyzed by wt AS-B, was determined to reach 35% of the total enzyme concentration. These studies are continued in this chapter which presents the measurement of the deacylation step for the wild-type enzyme, and the effect of mutations on the steady-state concentration of the thioester and its deacylation rate.

Materials and Methods

Enzymes and reagents

The construction of mutants in which asparagine-74 is replaced by alanine (N74A) or arginine-30 is replaced with an alanine (R30A) has been described previously (Boehlein et al., 1994b) Recombinant AS-B, N74A, and R30A were expressed and purified using standard procedures (Boehlein et al., 1994a). [U-^{14}C]-L-Glutamine (277 mCi/mmol) was purchased from Amersham. The nitrocellulose filters were purchased from Bio-Rad. Other reagents including L-glutamine were purchase from SIGMA Chemical Company (St. Louis, MO) and were of the highest commercial purity.

Effect of Glutamine on the Steady-State Concentration of the Thioester Intermediate

The radiolabeled enzyme complex was isolated by filter binding as described in chapter 5. A glutamyl-enzyme complex was formed by incubating 0.74 nmol of either wt, N74A, or R30A with various concentrations of L-glutamine (SA 22,000 dpm/nmol) in a 100 µl reaction containing 100 mM Tris-HCl (pH 8.0) for 30 sec at 5°C. The reactions were quenched in 1 ml of 8% TCA and 100 µl BSA (10

mg/ml) was added. After 2 minutes in the quench solution the samples were filtered by vacuum through a 2.5 cm nitrocellulose filter (0.45 mm porosity) and the filter was washed with 50 ml of 1N HCl. The filter was transferred to 5 ml of scintillation fluid (ScintiVers II*) and the ^{14}C activity was measured.

Rate of deacylation of the thioester intermediate

A glutamyl-enzyme adduct was formed at 5°C by incubating 0.74 nmol of either wt, N74A, or R30A with 1.5 mM L-glutamine (SA 20,000 dpm/nmol) in a 100 µl reaction containing 100 mM Bis-Tris and Tris (pH 8.0). After incubating the reaction for 1 min, 100 µl of 200 mM non-radioactive glutamine was added to each sample to dilute the unreacted radioactive glutamine. The samples were quenched at various time intervals after the dilution as described above and the amount of radioactivity associated with the protein was determined by scintillation counting.

Results

Characterization of the deacylation of wild-type AS-B

Isolation of a thioester intermediate formed between wild-type AS-B and glutamine and the steady-state concentration of this intermediate have been described in chapter 5.

A plot of thioester concentration versus time of incubation with unlabeled glutamine followed a mono-exponential decay and the rate constant for breakdown of the thioester determined from this plot was 0.16 sec^{-1} (Figure 6.1). This value is four times higher than the k_{cat} (Table 6.1).

Previous studies of the glutaminase reaction have indicated that the presence of ATP stimulates the hydrolysis of glutamine (Boehlein et al., 1994), therefore it was of interest to determine whether or not ATP affected the rate of the individual deacylation step. An examination of the effect of glutamine on the steady-state concentration of the thioester in the presence of 1 mM ATP suggests that ATP causes a slight decrease in the maximum concentration of thioester formed at saturating glutamine concentrations (Figure 6.2, Table 6.1). However, the addition of 1 mM ATP has no effect on the rate of deacylation (Figure 6.3, Table 6.1).

The effect of NH_4Cl on the deacylation rate was examined. If the step involving C-N bond cleavage to release ammonia is readily reversible it is expected that addition of ammonia to the reaction would shift the equilibrium away from the thioester and thus result in a decrease in the concentration of thioester formed and the rate of its deacylation. However, measurement of the deacylation of the thioester intermediate showed that the presence of 10 mM NH_4Cl had little effect on the rate of deacylation (Figure 6.4, Table 6.1).

The effect of glutamine on the steady state concentration of the glutamyl-enzyme adduct formed with the AS-B mutant, R30A

Studies in chapter 4 demonstrated that the rate limiting step of glutamine hydrolysis catalyzed by an AS-B mutant impaired in glutamine binding, R30A, was deacylation. Therefore, it would be expected that the deacylation rate would be the same as the k_{cat} value determined in glutamine hydrolysis. This comparison could be used in contrast to the slightly varying rates obtained for the wild-type enzyme. Unfortunately, the measurement of the deacylation

constant was impractical due to the high concentration and therefore the high specific activity of glutamine necessary to saturate this mutant enzyme. However, it was possible to examine the effect of glutamine on the steady-state concentration of the thioester formed with R30A. As shown in Figure 6.5 and Table 6.1, the maximum percentage of R30A which was labeled was higher than that obtained with the wild-type enzyme.

Characterization of a glutamyl-enzyme adduct formed with the AS-B mutant N74A

Examination of the effect of glutamine concentration on the steady-state level of the glutamyl-enzyme complex formed with N74A revealed a saturation curve which gave a maximum percentage of enzyme labeled with radioactivity of 57% and a K_m of 0.06 mM for glutamine (Figure 6.6 and Table 6.1). The thioester concentration in reactions using N74A followed a mono-exponential decay when plotted against time of incubation with unlabeled glutamine. The rate constant for deacylation determined from this plot was 0.062 sec^{-1}, significantly slower than that of wild-type (Figure 6.7 and Table 6.1).

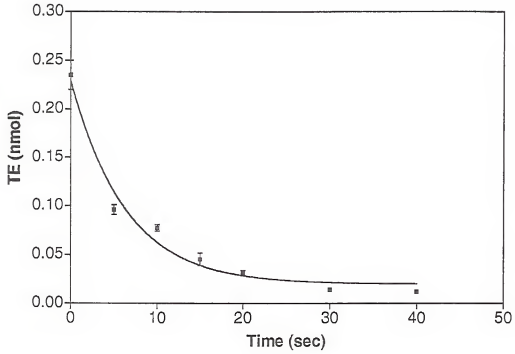


Figure 6.1: Rate of deacylation of the thioester intermediate formed during wild-type AS-B catalyzed glutamine hydrolysis.

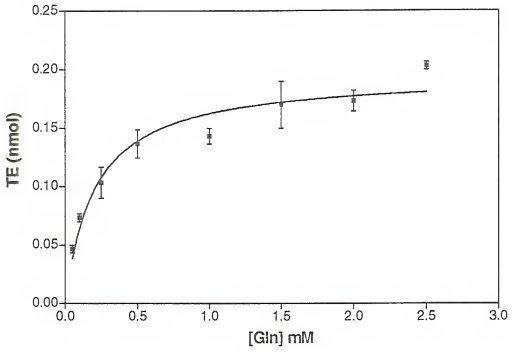


Figure 6.2: The steady-state concentration of the thioester formed in the presence of ATP.

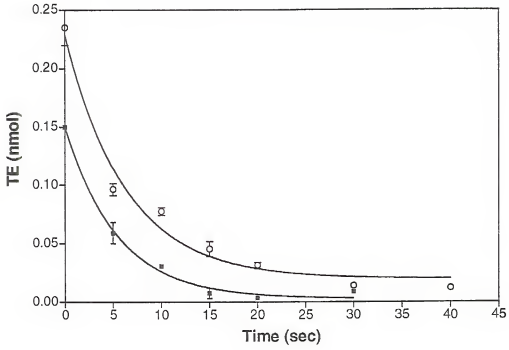


Figure 6.3: Effect of ATP on the rate of deacylation. A glutamyl-enzyme adduct was formed at 5°C by incubating 0.74 nmol wild-type AS-B with 1.5 mM glutamine (SA 20,000 dpm/nmol) and 1 mM ATP, ○ in 100 mM Bis-Tris and Tris (pH 8.0). After incubating the reaction for 1 min, the radioactive glutamine was diluted by the addition of 200 mM non-radioactive glutamine. The samples were quenched at various time intervals after the dilution as described in materials and methods and the amount of radioactivity associated with the protein was determined by scintillation counting. The deacylation in the presence of ATP, ○, was compared to that in the absence of ATP, ■.

Table 6.1: Characterization of the glutamyl-enzyme intermediate.

Enzyme	Max % of Enzyme labeled (%)	Deacylation rate (sec^{-1})	k_{cat} (sec^{-1})
wt	35.1 ± 0.9	0.16 ± 0.02	0.043 ± 0.0007
wt + 1 mM ATP	26 ± 1.1	0.18 ± 0.01	0.076 ± 0.002
wt + 10 mM NH_4Cl	nd	0.11 ± 0.01	nd[2]
R30A	50 ± 3	nd[1]	0.042 ± 0.001
N74A	57 ± 1	0.062 ± 0.005	0.024 ± 0.0005

All values in the table were determined at 5°C. [1]The deacylation rate for the reaction catalyzed by R30A could not be obtained due to the high concentrations of glutamine necessary to saturate the enzyme. [2]Ammonia has been shown to have little effect on the k_{cat} for the glutaminase reaction catalyzed by wt AS-B at 37°C.

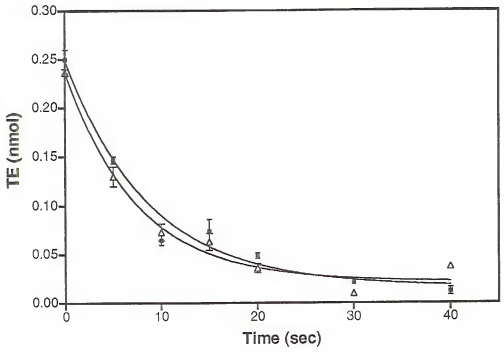


Figure 6.4: Effect of ammonia on the rate of deacylation. A glutamyl-enzyme adduct was formed at 5°C by incubating 0.74 nmol wild-type AS-B with 1.5 mM glutamine (SA 20,000 dpm/nmol) in 100 mM Bis-Tris and Tris (pH 8.0) in the presence, ■, and absence, ○, of 10 mM NH_4Cl. After incubating the reaction for 1 min, the radioactive glutamine was diluted by the addition of 200 mM non-radioactive glutamine. The samples were quenched at various time intervals after the dilution as described in materials and methods and the amounts of radioactivity associated with the protein was determined by scintillation counting.

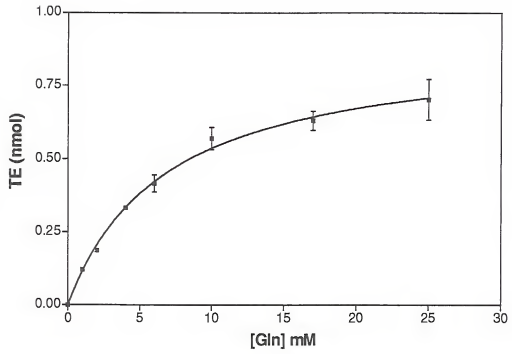


Figure 6.5: Effect of glutamine on the steady state concentration of thioester formed in the glutaminase reaction catalyzed by the AS-B mutant R30A. A glutamyl-enzyme complex was formed by incubating 1.8 nmol of R30A with various concentrations of L-glutamine (SA 22,000 dpm/nmol) in a reaction containing 100 mM Tris-HCl (pH 8.0) for 30 sec at 5°C. A radiolabeled enzyme complex was captured by quenching the reaction in 8% TCA over a nitrocellulose filter as described in materials and methods and the radioactivity associated with the filter was measured by scintillation counting.

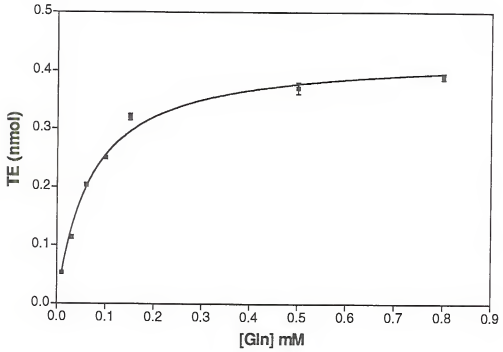


Figure 6.6: Effect of glutamine on the steady-state concentration of the thioester formed during the glutaminase reaction catalyzed by the AS-B mutant N74A. A glutamyl-enzyme complex was formed by incubating 0.74 nmol of N74A with various concentrations of L-glutamine (SA 22,000 dpm/nmol) in a reaction containing 100 mM Tris-HCl (pH 8.0) for 30 sec at 5°C. A radiolabeled enzyme complex was captured by quenching the reaction in 8% TCA over a nitrocellulose filter as described in materials and methods and the radioactivity associated with the filter was measured by scintillation counting.

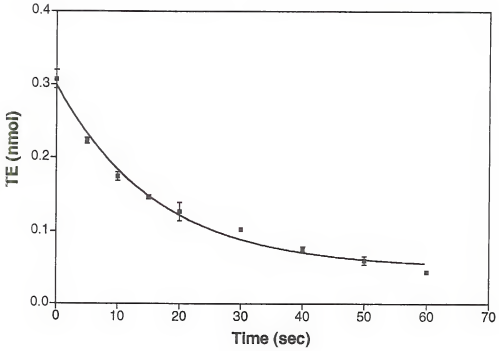


Figure 6.7: The rate of deacylation of the AS-B mutant N74A during glutamine hydrolysis. A glutamyl-enzyme adduct was formed at 5°C by incubating 0.74 nmol N74A with 1 mM glutamine (SA 20,000 dpm/nmol) in 100 mM Bis-Tris and Tris (pH 8.0). After incubating the reaction for 30 sec, the radioactive glutamine was diluted by the addition of 200 mM non-radioactive glutamine. The samples were quenched at various time intervals after the dilution as described in materials and methods and the amounts of radioactivity associated with the protein was determined by scintillation counting.

Discussion

Isolation of the thioester has allowed the examination of an individual step in glutamine hydrolysis, deacylation. The results presented here describe the use of this tool to show that the deacylation step is not solely rate determining in glutamine hydrolysis catalyzed by AS-B. The possibility of multiple steps making significant contributions to the overall rate rather than a single rate determining step was implied in chapter 4. In this chapter, not only was the deacylation rate 4-fold faster than k_{cat} for glutamine hydrolysis catalyzed by wild type AS-B but it was not affected by the presence of ATP (Table 6.1) which has been shown to stimulate the glutaminase reaction (Boehlein et al., 1994b). An increase in the rate of a reaction would require a change in rate in the slowest step or a step with a comparable rate to the slowest step of the reaction. Therefore, the above results suggest that a step in addition to deacylation is rate limiting and contributes to the overall rate constant for the reaction catalyzed by wild type AS-B. Further evidence for the lack of ATP involvement in the deacylation step comes from the absence of ATP stimulation of glutamine hydrolysis when deacylation is rate limiting such as the case which has been demonstrated for the glutaminase reaction catalyzed by the AS-B mutant, R30A (see chapter 4).

The characterization of the deacylation rate occurring in the AS-B catalyzed glutaminase reaction reveals some interesting contrasts in relation to amide hydrolysis catalyzed by the thiol protease papain and the amidotransferase. CAD (Chaparian & Evans,

1991). In support of the contrasts mentioned in chapter 4, CAD (Chaparian & Evans, 1991) and now AS-B have been shown to posses a different rate limiting step than found in amide hydrolysis catalyzed by papain. However, the present study suggests that while the deacylation step is the slowest step in glutamine hydrolysis catalyzed by CAD, AS-B catalyzed hydrolysis seems to have an additional step which has a rate comparable to deacylation. Secondly, high concentrations of ammonia essentially abolished the appearance of the thioester intermediate formed during CAD-catalyzed glutamine hydrolysis demonstrating that the acylation step is a reversible process (Chaparian & Evans, 1991). In contrast, ammonia seems to have a minimal effect on the AS-B-catalyzed thioester deacylation rate (Figure 6.4) which suggests a greater difficulty in reversing the formation of the thioester intermediate in the AS-B catalyzed reaction. Consistent with this observation, studies in chapter 3 show that ammonia does not inhibit in the range of concentrations used and that asparagine synthesis does not occur to a significant degree upon mixing the wild type enzyme with glutamate and ammonia. Hydroxylamine can be used in the synthesis of LGH therefore the lack of glutamine synthesis using ammonia is probably not due to the inability of glutamate to bind to the enzyme and form a thioester intermediate.

With the knowledge of the steady-state concentration of the thioester formed and the rate of deacylation in the glutaminase reaction catalyzed by wild type AS-B it is now possible to determine the involvement of individual residues in this step. This report describes an initial characterization of the deacylation step during

the glutaminase reactions catalyzed by R30A and N74A. While the deacylation rate was not obtainable for R30A, the steady state concentration of the thioester formed with this mutant was found to be slightly higher than that with the wild type enzyme which may be diagnostic of a slower deacylation step. Indeed, earlier studies provided evidence that the deacylation step was rate limiting for this mutant (partitioning studies in chapter 4). Furthermore, previous structural studies have indicated that the cognate arginine residue in GPA (Kim et al., 1996) and GFAT (Isupov et al., 1996), R26, is involved in positioning the conserved asparagine residue (N74 in AS-B). Since it is thought that this asparagine residue is important in forming an oxyanion hole in the stabilization of the tetrahedral intermediates, a decrease in deacylation step in the R30A catalyzed glutamine hydrolysis could reflect an indirect effect on the interactions made by this asparagine residue resulting in a decrease in stability of the second tetrahedral intermediate which occurs during deacylation. Further examination is necessary to confirm this idea. However, replacement of asparagine-74 with alanine (N74A) causes similar changes the amount of thioester formed and the deacylation rate (Table 6.1). Glutamine hydrolysis catalyzed by the N74A mutant, like R30A, exhibited a greater concentration of thioester than the wild type enzyme (Figure 6.6 & Table 6.1). Moreover, N74A exhibited a slightly slower rate of deacylation than the wild-type (Figure 6.7 & Table 6.1). Once again. this slower rate of deacylation may indicate the inability of an enzyme lacking this important asparagine to stabilize the second tetrahedral intermediate. Thus the deacylation step is slowed and with a lesser

change in the acylation step the amount of thioester that is formed increases.

The ability to measure the rate constants of the individual steps in glutamine hydrolysis will allow a complete characterization of the wild type enzyme and thus will provide a model of comparison for the determination of mutations on catalysis. This report represents the first step toward achieving this goal. The deacylation rate for the wild type AS-B now can be used as part of a model in the simulation of the glutaminase reaction presented in the following chapter. Furthermore, an extension of these studies using rapid quench analysis will provide rates of both acylation and deacylation thus furthering the development of the model and understanding the mechanism of glutamine hydrolysis.

CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

Understanding the mechanism of nitrogen transfer catalyzed by asparagine synthetase will require the knowledge of the integral parts of the process such as glutamine utilization. The past few years of research on AS-B have seen substantial progress towards this goal especially with respect to the catalytic roles played by the conserved residues in the glutamine active site (See Boehlein et al., 1994a,b; Boehlein et al., 1996; Boehlein et al., 1997a,b; Stoker et al., 1996) . From the knowledge gained in the investigations of AS-B and other class II amidotransferases, two schemes have developed which are used to describe glutamine utilization and nitrogen transfer. First, as described by Mei and Zalkin (1989) (see Figure 1.1), nitrogen transfer occurs via a free ammonia released from glutamine by a mechanism similar to thiol protease catalyzed amide hydrolysis. Alternatively, the nucleophilic attack of the N-terminal cysteine residue on the amide carbon of glutamine activates the side chain nitrogen for a direct attack on the acceptor molecule (Stoker et al., 1996) (see Figure 1.2). One key difference between these two schemes may lie in the requirement of a common mechanism for glutamine dependent asparagine synthesis and glutaminase reactions in the scheme involving free ammonia and the lack of a common mechanism for the two reactions in the scheme involving the direct

transfer of nitrogen. Therefore, one way to discriminate between which mechanism describes asparagine synthesis catalyzed by AS-B is to obtain a detailed model, including the rates of the individual steps, of both glutamine dependent reactions. Such a model also would expedite the course of understanding how individual amino acid residues participate in catalysis as it would allow us to visualize which individual step is affected by a mutation. Finally, a detailed model of the glutaminase reaction is necessary in order to complete the description of the overall kinetic mechanism of AS-B.

The goal of this dissertation was to define both chemical and kinetic characteristics of the glutaminase reaction which could aid in building this much needed model. Certainly as shown here, the initial stages are complete. Due to some similarities between the class II amidotransferases and the thiol proteases, such as the presence of a catalytically important cysteine, the original model for the glutaminase reaction was based on the mechanism of amide hydrolysis catalyzed by the thiol protease, papain. Therefore, as a first step, it was important to ascertain that the major features of this mechanism, such as the cys-his dyad and the formation of a thioester intermediate, could be found in AS-B catalyzed glutamine hydrolysis.

For papain, the importance of Cys-25 and His-159 is first apparent through their complete conservation among all members of the family of thiol proteases (Brocklehurst et al., 1987). The conserved histidine plays a number of essential roles in the hydrolysis reaction including activation of the Cys-25, protonation of the leaving group, and activation of a water molecule for nucleophilic

attack on the thioester (Storer & Menard, 1994). If there is a histidine playing an equivalent role in AS-B catalysis, then it would be expected that its removal would not only dramatically affect the rate of the glutaminase reaction but would also change the pH profile. However, as shown in chapter 2, replacing histidine-47 with an asparagine only causes a four-fold decrease in the k_{cat} for glutamine hydrolysis. Moreover, the pH profile of the glutaminase reaction catalyzed by this mutant is similar to that of the wild-type. These results suggest that a role for His-47 in acid-base catalysis is unlikely. On the other hand, the 10-fold drop in activity should not be ignored, especially in light of the hypothesis presented here that a conformation change is rate limiting. One further test for the participation of His-47 in acid-base capacity would be to examine the individual steps catalyzed by H47N. The work presented in this dissertation has provided a method to examine the deacylation step in the glutaminase reaction. Therefore, a comparison of the pH-dependence of the deacylation rate catalyzed by wild-type AS-B and its H47N mutant might provide the information necessary to reveal whether His-47 plays a role in activating a water molecule for a nucleophilic attack on the thioester.

While there may be differences in acid-base catalysis between AS-B and papain, the overall pathway of hydrolysis may be similar. This means that there must be an alternative for the deprotonation of Cys-1 and the protonation of the leaving group during hydrolysis catalyzed by AS-B. In fact, such an alternative has been offered in regards to the class II amidotransferases (Kim et al., 1996). Structural studies have placed the class II amidotransferases in a

family of hydrolytic enzymes, the Ntn-hydrolases, which use the N-terminal residue (Ser, Thr, or Cys) as the nucleophilic catalyst. Members of this family, including the 20S proteasome (Brannigan et al., 1995; Seemuller et al., 1996), aspartylglucosaminidase (Oinonen et al., 1995), and penicillin acylase (Duggleby et al., 1995), are thought to catalyze general acid-base reactions through their N-terminal amino group. Whether or not the class II amidotransferases use such a mechanism remains to be established since previous studies, employing site-directed mutants of GPA and GFAT which contain an additional residue N-terminal to the cysteine, have given ambiguous results (Isupov et al., 1996; Kim et al, 1996). Another alternative for a histidine could be specific acid-base catalysis. Indeed, this mechanism would explain the unexpected differences between the V/K ^{15}N KIEs for the glutaminase reaction catalyzed by AS-B (Stoker et al., 1996) and that for amide hydrolysis catalyzed by papain (O'Leary et al., 1974). For papain, theoretical studies have suggested that the transfer of the proton from His-159 to the leaving group amine is concerted with the nucleophilic attack of Cys-25 (Arad et al., 1990). While this conclusion remains to be confirmed, the large V/K ^{15}N KIEs implied that C-N bond cleavage was the slow step in the acylation process. On the other hand, the absence of a V/K ^{15}N KIE in the glutaminase reaction catalyzed by AS-B suggested that a step during acylation other than C-N bond cleavage was rate limiting. This observation may reflect a slow protonation of the leaving group regulated by a specific acid catalyst. This issue could be addressed by a study of the pH dependence of the ^{15}N V/K KIE. If the rate of acylation is dependent on the protonation of the leaving

group via specific acid catalysis, then the ^{15}N V/K KIE might increase at low pHs where it is easier to protonate the leaving group. The limitation to such a study is the narrow pH range in which AS-B is stable.

The finding that there are differences in acid-base catalysis between papain and AS-B raises the question of whether the AS-B catalyzed glutaminase reaction follows a pathway which has any similarities to that of papain. Therefore, the existence of an acyl-enzyme intermediate in the pathway of glutamine hydrolysis catalyzed by AS-B was investigated. Chapter 5 presents evidence for such an intermediate as the combination of [^{14}C] glutamine with the wild-type AS-B resulted in a radiolabeled enzyme which could be trapped by precipitation on a nitrocellulose filter. Evidence that the glutamyl-enzyme adduct is a thioester includes: 1) the inability to form a glutamyl-enzyme adduct with the AS-B mutant, C1A, which can bind glutamine but lacks the cysteine thought to make the thiol linkage (Chapter 5) 2) the inability to form an adduct with wild-type enzyme that had been incubated with a covalent modifier (DON) which was shown to be competitive with glutamine and to cause irreversible inhibition of the glutaminase activity (Chapter 4) 3) the observation that the amount of intermediate saturates with increasing glutamine concentration and the half saturation point is consistent with steady state parameters observed for the AS-B glutaminase reaction (Chapter 5), and 4) the instability of the intermediate in a basic environment as would be expected of a thiol ester intermediate (Chapter 5). Future studies to support the conclusion that the covalent intermediate is a thioester include the

examination of the enzyme structure in the presence of ^{15}N and ^{14}C double labeled glutamine using solid state nmr.

Computer Simulations of glutamate formation as a function of time:

The evidence for the existence of a thioester intermediate given in chapters 5 and 6 provides a starting point in building a model for the AS-B glutaminase reaction. In this dissertation the model has been built from the simplest case which involves the formation of a covalent intermediate (shown in scheme (1)), and additions have been made according to the minimal requirements necessary to make the best fit to the actual data from the examination of glutamate formation as a function of time.

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}TE \underset{+\,P_1}{} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \qquad (1)$$

In this model, k_1 and k_{-1} represent the steps leading to and from the formation of the Michaelis complex; k_2 and k_{-2} the steps involved in the acylation process; and k_3 and k_{-3} the steps involved in deacylation. The experiments in chapters 4 and 6 yielded values for several of the rate constants in this reaction which now can be used in a simulation of the glutaminase reaction using the computer program KINSIM (Barshop et al. 1983). The values for the rate constants to be used in the simulation of scheme (1) are given in Table 7.1. The dissociation constant (K_s) for glutamine can be used to obtain the value of k_{-1} using the relationship, $K_s = k_{-1}/k_1$ and assuming the value of k_1 approaches diffusion. The direct measurement of

deacylation demonstrated in chapter 6 provides a rate constant for k_3. The observation of a burst in glutamate formation with time presented in chapter 4 suggests that k_3 is less than k_2 and therefore k_2 will be arbitrarily set at 1 sec^{-1}. Preliminary studies using rapid quench techniques have confirmed that this value of k_2 is reasonable. The acylation step will be made irreversible since the backwards reaction seems unlikely. Recall that ammonia has no effect on the rate of deacylation (chapter 6, Figure 6.4), it does not seem to act as a substrate in the synthesis of glutamine (chapter 3), and it does not inhibit the glutaminase reaction (chapter 3). The value for k_{-3} is estimated from the observation that the rate of LGH synthesis is 12% of the rate of its hydrolysis. Partitioning studies suggest that hydroxylamine readily attacks the thioester, therefore it is probably not the rate determining step for the reverse reaction. Rather, the step most likely to govern the rate of the reverse reaction is formation of the thioester from glutamate (k_{-3}). Thus, an upper limit can be set to 12% of the forward rate for k_{-3}. However, the value of k_{-3} was varied in order to attempt to fit the simulation to the actual data. These values were applied to a simulation of the formation of glutamate with time. It is apparent from the fit shown in Figure 7.1 that scheme (1) is not sufficient to describe the experimental data. First, such a scheme results in the lack of a burst in glutamate formation. Second, the slope of glutamate formation as a function of time and thus k_{cat} are too high in the simulated data when compared to the actual values (see Table 7.4).

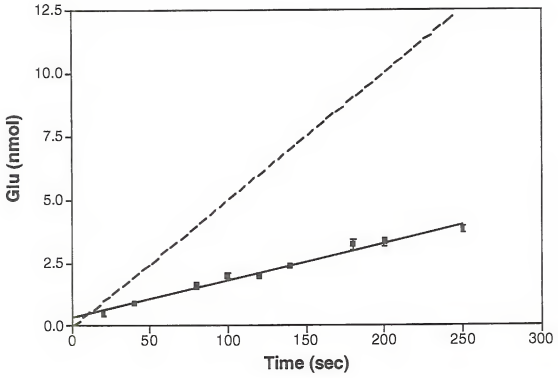


Figure 7.1: Comparison of the simulated to the actual data representing glutamate formation as a function of time. The dashed line (---) represents the simulation of equations (1) and (2). The solid line (—) that parallels the actual data points (■) represents the simulation from equation (3).

Table 7.1: Kinetic parameters at 5°C for glutamine hydrolysis catalyzed by wild type AS-B according to schemes (1) and (2).

step	forward (k_n)	reverse (k_{-n})
1	$1x10^{8}$ $M^{-1}sec^{-1}$	$1.75x10^{4}$ sec^{-1}
2	1 sec^{-1}	$1x10^{-6}M^{-1}$ sec^{-1}
3	$1.6x10^{-1}$ sec^{-1}	$1.6x10^{-2}$ sec^{-1}
4	$1.82x10^{7}$ sec^{-1}	$1x10^{8}$ $M^{-1}sec^{-1}$

Steps 1 through 3 were used in the simulation of scheme (1) whereas all 4 steps were used in scheme (2).

The examination of the deacylation of the thioester, presented in chapter 6, provided evidence that the deacylation was not the sole rate determining step. Therefore, the additional step which may influence the overall rate of the reaction should be added to the model above. The knowledge of both the deacylation rate and the K_i for glutamate obtained in this work allow this expansion of the minimal model to include both deacylation and product release as separate steps as shown in scheme (2):

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{+\,P_1}{E\text{-}TE} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_2 \qquad (2)$$

where EP_2 represents the enzyme-glutamate complex and step 4 represents product release. For the simulation of this model, steps 1-3 were set at the same values as in scheme (1) (Table 7.1) and k_{-3} was varied. The value for k_4 was derived (Table 7.1) from the K_i for glutamate assuming that k_{-4} approaches diffusion just as the case for k_1.

The high value for K_i leads to the calculation of a very fast product release, therefore the extra step added to the model had little effect on the fit of the simulated values to the actual data (Figure 7.1). A much more precise fit of the data was obtained by adding an additional step to account for a slow conformational change prior to the fast product release as shown in scheme (3) below:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}TE \underset{+P_1}{} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} FP_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + P_2 \qquad (3)$$

The rate constants of the first three steps of this scheme are the same as in scheme (2) (Table 7.2). The inhibition constant for glutamine will now be used in step 5 instead of step 4. The value for k_{-4} was arbitrarily set to 1 sec^{-1}. Results in chapter 4 suggest that another step occurring after the formation of the thioester intermediate may be rate limiting in glutamine hydrolysis. In scheme (3) the slow step could either be k_4 or k_5. Since inhibition studies and the simulation of scheme (2) rule out k_5 as being slow, k_4 was given the slowest rate of scheme (3) (Table 7.2) with k_{cat} setting the lower limit to 0.04 sec^{-1}. As shown in figure 7.1 and Table 7.3, these estimates suggest that scheme (3) is sufficient to describe glutamine hydrolysis catalyzed by wild-type AS-B.

The work in this dissertation has been combined to form a minimal model of the glutamine hydrolysis reaction in which the formation of the thioester is fast relative to its breakdown, and in which two steps (deacylation and rate limiting conformational change) regulate the rate of the reaction. This model will provide a foundation on which to begin forming a complete description of the reaction mechanism. In fact, efforts are now underway to obtain experimental values using rapid quench techniques for some of the estimated rate constants (such as k_2) in this model. Rapid quench combined with the thioester trapping techniques described in chapter 5 could be used to compare the rates of thioester formation

Table 7.2: Kinetic parameters at 5°C for glutamine hydrolysis catalyzed by AS-B according to equation (3).

step	forward (k_n)	reverse (k_{-n})
1	$1x10^{8}$ $M^{-1}sec^{-1}$	$1.75x10^{4}$ sec^{-1}
2	1 sec^{-1}	$1x10^{-6}$ M^{-1} sec^{-1}
3	$1.6x10^{-1}$ sec^{-1}	$1.6x10^{-2}$ sec^{-1}
4	$7x10^{-2}sec^{-1}$	1 sec^{-1}
5	$1.82x10^{7}$ sec^{-1}	$1x10^{8}$ M^{-1} sec^{-1}

Table 7.3: Comparison of the actual and simulated values obtained for the k_{cat}, slope, intercept, and [TE] from Figure 7.1.

	Experimental values	Simulated Values		
		(1)	(2)	(3)
k_{cat}	0.040	0.135	0.135	0.043
slope	0.015	0.050	0.050	0.016
Y-intercept	0.34	-0.04	-0.04	0.15
[TE]	2.51×10^{-6}	6.29×10^{-6}	6.29x10	2.46×10^{-6}

between the glutaminase and glutamine dependent asparagine synthesis reactions. If the mechanism of nitrogen transfer involves a direct attack of the amine of the tetrahedral intermediate on the acceptor substrate then, at least at high pH, the glutamine dependent asparagine synthesis reaction might have a faster acylation rate than the glutaminase reaction since this reaction would not necessarily require a protonation step.

Another feature of the reaction that will require experimental testing is the existence of a rate determining conformational change which has been predicted by the model. Studies are already underway to measure the rate of fluorescence change during AS-B catalyzed conversion of glutamine to glutamate using stop-flow techniques. If the rate is found to be consistent with a rate determining conformational change then it would be interesting to compare this rate with that for the AS-B mutant R30A since the reaction catalyzed by this mutant seems to have a different rate limiting step (see partitioning experiments in chapter 4).

The studies summarized here represent the first step towards completing a detailed chemical and kinetic description of glutamine hydrolysis catalyzed by AS-B. With further experimental support, the model developed in this work can be used as a basis for comparison in examining the effects of mutations on glutamine hydrolysis in efforts to determine the roles of the individual amino acid residues in the utilization of glutamine. Ultimately, these results may be significant in forming a bridge toward understanding the overall mechanism of nitrogen transfer.

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BIOGRAPHICAL SKETCH

Holly Schnizer was born on April 11, 1969 to Dr. Flint and Dottye Gray of Kingsport, Tennesse. In 1991, Holly received her Bachelor of Science degree in chemistry from Centre College in Danville, Kentucky. The following fall, Holly began her graduate studies at the University of Florida in the Department of Biochemistry and Molecular Biology under the supervision of Dr. Sheldon Schuster. In the spring of 1996, Holly married Dr. Richard Schnizer whom she met while working in the laboratory. She expects to receive her Ph.D. in Biochemistry and Molecular Biology in the fall of 1997.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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